PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
R. Sanders WILLIAMS *et al.*

Serial No.: 09/782,953

Filed: February 13, 2001

For: METHODS AND COMPOSITIONS RELATING TO MUSCLE SELECTIVE CALCINEURIN INTERACTING PROTEIN (MCIP)

Group Art Unit: 1656

Examiner: Liu, Samuel W.

Atty. Dkt. No.: MYOG:036US/SLH

Confirmation No.: 2337

CERTIFICATE OF ELECTRONIC TRANSMISSION
37 C.F.R. § 1.8

I hereby certify that this Appeal Brief is being electronically filed with the United States Patent and Trademark Office via EFS-Web on the date below:

May 6, 2008
Date

Steven L. Highlander

APPEAL BRIEF

TABLE OF CONTENTS

Page

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: R. Sanders WILLIAMS *et al.*	Group Art Unit: 1656
Serial No.: 09/782,953	Examiner: Liu, Samuel W.
Filed: February 13, 2001	Atty. Dkt. No.: MYOG:036US/SLH
For: METHODS AND COMPOSITIONS RELATING TO MUSCLE SELECTIVE CALCINEURIN INTERACTING PROTEIN (MCIP)	Confirmation No.: 2337

APPEAL BRIEF

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-01450

Dear Sir:

This Appeal Brief is filed in response to the final Office Action mailed on August 6, 2007. Appellant's brief is due on May 6, 2008, by virtue of the Notice of Appeal filed on December 6, 2007, and the accompanying Petition for Extension of Time (three months) and payment of fees. Also included herewith is the fee for the brief. No other fees are believed due in connection with this filing; however, should appellant's payment be missing or deficient, or should any fees be due, the Commissioner is authorized to debit Fulbright & Jaworski L.L.P. Deposit Acct. No. 50-1212/MYOG:036US/SLH.

I. Real Party In Interest

The real parties in interest are the assignee, the Board of Regents, University of Texas System, Austin, TX, and the licensee, Gilead Colorado, Westminster, CO.

II. Related Appeals and Interferences

There are no related appeals or interferences.

III. Status of the Claims

Claims 1-101 were filed with the original application. Claims 1-58, 60, 63-69 and 71-101 have been canceled, and thus claims 59, 61, 62 and 70 are pending, stand rejected, and are appealed. A copy of the appealed claims is attached as Appendix A.

IV. Status of the Amendments

No amendments have been offered after mailing of the final Office Action.

V. Summary of the Claimed Subject Matter

Claim 59 is drawn to a method of modulating muscle cell growth in striated muscle cells in a human subject comprising (a) identifying a human subject in need of striated muscle cell growth modulation; (b) selecting a small molecule for its ability to modulate Muscle Selective Calcineurin Interacting Protein (MCIP1) expression; and (c) administering said small molecule to said human subject, whereby administration of said modulator results in modulation of striated muscle cell growth in said human subject. The claim is supported in the specification as filed at page 6, lines 24 to 27, and page 7, line 2.

VI. Ground of Rejection to be Reviewed on Appeal

A. Is claim 70 indefinite under the second paragraph of §112?

B. Are claims 59, 61 and 70 anticipated §102(b) over U.S. Patent 4,330,557 ("the '557 patent"; Exhibit 1)?

C. Are claims 59 and 62 anticipated under §102(b) over U.S. Patent 5,651,980 ("the '980 application"; Exhibit 2)?

D. Are claims 59, 62 and 70 anticipated under §102(b) over U.S. Patent 5,958,404 ("the '404 patent"; Exhibit 3)?

VII. Argument

A. Standard of Review

Findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. §706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by "substantial evidence" within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *Gartside*, the Federal Circuit stated that "the 'substantial evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* at 1312. Accordingly, it necessarily follows that an examiner's position on appeal must be supported by "substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

B. Rejection Under 35 U.S.C. §112, Second Paragraph

The examiner has rejected claim 70 under the second paragraph of §112 as being indefinite.[1] More specifically, claim 70 is rejected over use of the term "second pharmaceutical agent." The examiner argues that it is unclear whether this second agent is the same as or different than the small molecule of claim 59. Appellants previously attempted to amend claim 59 to specify a "first" agent in that claim so as to further point out that the two agents are distinct, but entry of this amendment was denied, and appellants remain willing to provide such an amendment, or authorize such an examiner's amendment, but even in the absence of such, the present claim, phrased in terms of "further comprising administering ... a second pharmaceutical agent," is more than clear in differentiating the agents. Reversal of the rejection is therefore requested.

C. Rejections Under 35 U.S.C. §102(b)

i. U.S. Patent 4,330,557

Claims 59, 61 and 70 are rejected under 35 U.S.C. §102(b) over U.S. Patent 4,330,557 ("the '557 patent"; Exhibit 1). Appellants traverse.

The examiner argues that because certain fatty acids activate calcineurin, and calcineurin activates MCIP, the '557 patent anticipates the appealed claims. What is missing from this analysis, however, is the ***knowledge*** that calcineurin regulates MCIP, which can only be found in appellants' specification. In order to make the deficiency in the '557 patent more clear, appellants have amended the claims to more precisely state that the small molecule is selected on the basis of its MCIP-modulating function. The cited reference clearly fails to provide

[1] The §112, second paragraph rejection of claim 59, though not stated as withdrawn at page 2 of the final Office Action, is not discussed at pages 2-3, and thus is presumed to have been withdrawn.

information necessary to accomplish this step (inherent functions of calcineurin notwithstanding), and therefore the rejection is improper and its reversal is requested.

ii. U.S. Patent 5,651,980

Claims 59 and 62 are rejected under 35 U.S.C. §102(b) over U.S. Patent 5,651,980 ("the '980 application"; Exhibit 2). Appellants traverse.

The examiner argues that because cyclosporin inhibits calcineurin, and calcineurin activates MCIP, the '980 patent anticipates the appealed claims. What is missing from this analysis, however, is the ***knowledge*** that calcineurin regulates MCIP, which can only be found in appellants' specification. In order to make the deficiency in the '980 patent more clear, appellants have amended the claims to more precisely state that the small molecule is selected on the basis of its MCIP-modulating function. The cited reference clearly fails to provide information necessary to accomplish this step (inherent functions of calcineurin notwithstanding), and therefore the rejection is improper.

Moreover, the reference fails to teach or suggest the step of "identifying a human subject in need of striated muscle cell growth modulation" as the '980 patent deals with patients suffering from ***inflammatory responses in transplant scenarios***. Thus, for a second reason, the rejection is improper, and therefore its reversal is requested.

iii. U.S. Patent 5,958,404

Claims 59, 62 and 70 are rejected under 35 U.S.C. §102(b) over U.S. Patent 5,958,404 ("the '404 patent"; Exhibit 3). Appellants traverse.

The examiner argues that because cyclosporin inhibits calcineurin, and calcineurin activates MCIP, the '404 patent anticipates the appealed claims. What is missing from this analysis, however, is the ***knowledge*** that calcineurin regulates MCIP, which can only be found in

applicants' specification. In order to make the deficiency in the '404 patent more clear, applicants have amended the claims to more precisely state that the small molecule is selected on the basis of its MCIP-modulating function. The cited reference clearly fails to provide information necessary to accomplish this step (inherent functions of calcineurin notwithstanding), and therefore the rejection is improper.

Moreover, the reference fails to teach or suggest the step of "identifying a human subject in need of striated muscle cell growth modulation" as the '404 patent deals with patients suffering from ***inflammatory responses in transplant scenarios***. Thus, for a second reason, the rejection is improper, and therefore its reversal is requested.

D. Conclusion

In light of the foregoing, appellant respectfully submits that all pending claims definite, novel and non-obvious. Therefore, it is respectfully requested that the Board reverse each of the pending rejections.

Respectfully submitted,

Date: May 6, 2008

Steven L. Highlander
Reg. No. 37,642

Fulbright & Jaworski L.L.P.
600 Congress Ave., Suite 2400
Austin TX 78701
512-474-5201

VIII. APPENDIX A – APPEALED CLAIMS

59. A method of modulating muscle cell growth in striated muscle cells in a human subject comprising:

(a) identifying a human subject in need of striated muscle cell growth modulation;
(b) selecting a small molecule for its ability to modulate Muscle Selective Calcineurin Interacting Protein (MCIP1) expression; and
(c) administering said small molecule to said human subject,

whereby administration of said modulator results in modulation of striated muscle cell growth in said human subject.

61. The method of claim 59, wherein said small molecule is an agonist of muscle cell growth.

62. The method of claim 59, wherein said small molecule is an antagonist of muscle cell growth.

70. The method of claim 59, further comprising administering to said human subject a second pharmaceutical agent used to treat cardiac disease.

IX. APPENDIX B – EVIDENCE CITED

Exhibit 1 – U.S. Patent 4,330,557

Exhibit 2 – U.S. Patent 5,651,980

Exhibit 3 – U.S. Patent 5,958,404

X. APPENDIX C – RELATED PROCEEDINGS

None

EXHIBIT 1

United States Patent [19]

Cavazza

[11] 4,330,557

[45] May 18, 1982

[54] **ACYL-CARNITINE AND USE THEREOF IN PARENTERAL ADMINISTRATION OF TRIGLYCERIDES**

[76] Inventor: **Claudio Cavazza**, 35, Via Marocco, 00144 Rome, Italy

[21] Appl. No.: **130,801**

[22] Filed: **Mar. 17, 1980**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 41,147, May 21, 1979, abandoned.

[51] **Int. Cl.**[3] **A61K 31/205**

[52] **U.S. Cl.** **424/316**

[58] **Field of Search** 424/316

[56] **References Cited**

U.S. PATENT DOCUMENTS

3,793,450	2/1974	Schnell	424/343
3,810,994	5/1974	Wiegand	424/316

Primary Examiner—Stanley J. Friedman

Attorney, Agent, or Firm—Ladas & Parry

[57] **ABSTRACT**

A composition for total or supplemental parenteral nutrition of patients in need thereof for the treatment of shock and trauma is disclosed. The composition comprises a therapeutically effective amount of triglycerides and an amount of an acyl-carnitine, typically acetyl-carnitine, or a pharmaceutically acceptable salt thereof sufficient to increase free fatty acid oxidation.

14 Claims, No Drawings

ACYL-CARNITINE AND USE THEREOF IN PARENTERAL ADMINISTRATION OF TRIGLYCERIDES

CROSS-REFERENCE

This is a continuation-in-part of Ser. No. 041,147 filed May 21, 1979, now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to a novel pharmaceutical composition for total or supplemental parenteral nutrition of patients in need thereof for treatment of shock and other trauma.

More particularly, the present invention relates to a novel composition for use in total or supplemental intravenous nutrition of patients in need thereof, such composition comprising a therapeutically effective amount of triglycerides. The present invention also relates to a therapeutical method of increasing the efficiency of triglyceride administration to patients in need thereof because of their condition of shock and trauma.

2. Description of the Prior Art

Up until recently, total parenteral nutrition was limited to the use of carbohydrates and protein hydrolysates, whereas intravenous administration of fats as caloric source was actually avoided in spite of its acknowledged utility based on the long-standing knowledge that several tissues, particularly the muscular tissue and the myocardium, utilize fatty acids as preferential energy substrate. Consequently, intravenous administration of exogenous fats would result in markedly beneficial effects in all those clinical situations wherein unbalanced conditions of some metabolic systems may occur.

One of these systems is for instance the adjustment system of the lipolysis whose role is that of furnishing suitable material to the tissues which utilize fatty acids and ketone bodies as energy source.

A second system, more closely related to the phenomena of the mitochondrial respiration, is the system wherein the carnitine-acetyl carnitine transferase complex plays an essential role. This complex is strictly related to the activity of ATP mitochondrial translocase and acts so as to allow the passage of the activated, long-chain free fatty acids through the mitochondrial membrane to take place and their attendant conveyance to the beta-oxidation sites.

The consequence brought about by the alterations of the abovementioned systems is the intracellular accumulation of long-chain fatty acids which, therefore, cannot be properly utilized. The muscular cells are thus deprived of an energy substrate of the utmost importance and muscular proteolysis is thereby enhanced with attendant loss of branched-chain aminoacids which are utilized by the muscular tissues for energy purposes. This impaired free fatty acid utilization causes the blockage of several enzyme systems of the mitochondrial walls and the onset of cardiac rhythm disturbances.

More recently, administration of triglycerides to patients affected by conditions of shock and trauma has become a problem of major concern and after extensive experimentation and researches lipid packs in the form of an intravenous emulsion of fats and oils have been developed and become commercially available. As an instance of useful lipid pack, Intralipid (marketed by Cutter Laboratoires, Berkeley, Calif.) can be cited. Intralipid is made up of 10% soybean oil (a mixture of the glycerides of oleic, linolic, palmitic, stearic and linolenic acids), 1.2 egg yolk phospholipids, 2.25% glycerin, the balance being water for injection, sufficient sodium hydroxide being added to adjust the pH to 5.5–9.0.

Other compositions of lipid packs at present available on the market are disclosed in "Total parenteral nutrition" by Parshotam L. Madan, Devendra K. Madan and Joseph F. Palumbo, Drug Intelligence and Clinical Pharmacy, Vol 10, Dec. 76, pages 684–696, and in "L'alimentation parenterale par émulsions lipidiques" by G. Duchesne, La Revue du Praticien (1974), 24, 5, pages 377–384. The disclosures of these articles are incorporated by reference in this specification.

Exogenous triglycerides of the intravenous fat emulsion are intended to be hydrolyzed in the body by lipase with attendant formation of glycerol and fatty acids. These latter should in turn undergo progressive oxidation.

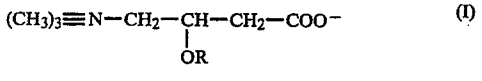
SUMMARY OF THE INVENTION

It has been found however that the administration of lipid packs to patients in need of exogenous triglycerides frequently does not lead to therapeutically satisfactory results because of the sharply reduced clearance of triglycerides and free fatty acids by patients in shock and trauma, particularly by intensively catabolic patients (such as, e.g. those who have undergone extensive burns, fractures or major surgical operations). Reduced clearance of triglycerides and free fatty acid has been recently shown to occur also in premature and small for gestational age babies.

It is, therefore, one object of the present invention to provide a pharmaceutical, triglyceride-comprising composition which allows the efficiency of exogenous triglycerides administered to patients for treatment of shock and trauma to be increased.

It is a further object of the present invention to provide a triglyceride-comprising composition suitable to minimize or prevent adverse metabolic reactions to exogenous triglycerides, such as the depletion of endogenous carnitine in the heart and other muscular tissues that can take place as a consequence of lipid pack administration.

In accordance with the present invention, there has now been discovered a parenterally administrable pharmaceutical composition useful for providing nutrition to humans comprising an amount of physiologically acceptable triglycerides therapeutically effective for nourishing said humans, an amount of an acyl carnitine of the general formula



(I)

wherein R represents acetyl, propionyl, butyryl, hydroxybutyryl, hexanoyl, octanoyl, decanoyl, palmitoyl, stearoyl, acetoacetyl, succinyl, isovaleryl and crotonyl or a pharmaceutically acceptable salt thereof, sufficient to enhance free fatty acid oxidation, and a pharmaceutically acceptable carrier therefor.

The amount of the acyl-carnitine of formula (I) or the pharmaceutically acceptable salt thereof is from about 2 to about 30 g/l of parenterally administrable composition.

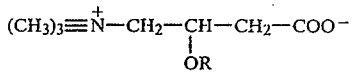
It has been found that exogenous acyl-carnitine of formula (I) enhances the efficiency of triglyceride administration, because acylcarnitine of formula (I) increases the oxidation rate of free fatty acids derived from exogenous triglycerides parenterally administered to patients for treatment of shock and trauma.

It has in fact been found that exogenous acyl-carnitine of formula (I) is suitable for re-activating the mitochondrial respiration processes because it supplies energy-releasing material (i.e. the acyl groups) which has direct access to the krebs cycle, and allows the passage of the long chain fatty acids through the mitochondrial membrane to be resumed, thus making possible the beta-oxidation processes to get started again.

It has furthermore been found that co-administration of exogenous acyl-carnitine of formula (I) and exogenous triglycerides is suitable to counterbalance or prevent the carnitine depletion in the muscular tissues, particularly in the myocardium, which can be brought about by the administration of lipid packs to patients in need thereof for the treatment of shock and trauma.

It should be understood that, within the scope of this invention, by the term "triglycerides" those glycerides are meant which are pharmacologically acceptable to a human and, furthermore, are of significance as far as the lipid requirements in the human diet are concerned.

The present invention also provides a method of treating patients in need of total parenteral nutrition, which comprises parenterally administering to a patient in need thereof an amount of physiologically acceptable triglycerides therapeutically effective for nourishing said patient and in combination therewith an amount of acyl-carnitine of the general formula



wherein R represents acetyl, propionyl, butyryl, hydroxy butyryl, hexanoyl, octanoyl, decanoyl, palmitoyl, stearoyl, acetoacetyl, succinyl, isovaleryl and crotonyl or a pharmaceutically acceptable salt thereof sufficient to enhance free fatty acid oxidation.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

It has been found that lipid components particularly suitable for use in combination with the acyl-carnitines of formula (I) or a pharmaceutically acceptable salt thereof in the parenterally administrable compositions of this invention are soybean oil, cottonseed oil, sesame oil and safflower oil.

As known, these oils contain the glycerides of linoleic, oleic, linolenic and palmitic acids, the relative amounts of the various glycerides varying with the specific oil considered.

It is apparent that other pharmacologically acceptable, edible oils which comprise the foregoing glycerides may be used in the compositions of the present invention.

It has been also found that particularly suitable compositions for the total parenteral mutrition in accordance with the present invention are as follows:

Composition 1	
soybean oil	50-200 g/l
glycerine	22-26 g/l
egg yolk phospholipids (ovolecithin)	10.5-12.5 g/l
acetyl-carnitine or pharmaceutically acceptable salt thereof	5-25 g/l
sodium hydroxide	sufficient to adjust the pH of the composition to 5.5-9.0
distilled water	balance to 1 liter.

The composition thus obtained is an isotonic intravenous emulsion having an osmolarity of from about 250 to 330 milliosmoles/kg of distilled water.

Composition 2	
cottonseed oil	100-200 g/l
soybean lecithin	10-30 g/l
sorbitol	40-60 g/l
D,L-α-Tocopherol	0.5-1 g/l
acetyl-carnitine or pharmaceutically acceptable salt thereof	5-25 g/l
Distilled water	Balance to 1 liter

Composition 3	
Sesame oil	100-200 g/l
Glycerine	20-30 g/l
Cetylstearylsulfonic acid	1-1.5 g/l
Propionyl-carnitine or pharmaceutically acceptable salt thereof	3-20 g/l
Distilled water	Balance to 1 liter

Composition 4	
Safflower oil	100-200 g/l
Sorbitol	50-70 g/l
Polysorbate	8-12 g/l
D,L-α-Tocopherol	0.5-1 g/l
Acetoacetyl-carnitine or pharmaceutically acceptable salt thereof	2-20 g/l
Distilled water	Balance to 1 liter

The desired daily dosage of the composition will be determined in accordance with standard usage, a daily dosage of 500 ml being generally sufficient.

According to a preferred embodiment, the method of treating patients in need of total parenteral nutrition comprises administering first the above specified emulsions and continuing carnitine administration for a total of 12 to 24 hours, after discontinuation of said triglyceride administration.

This will insure that sufficient carnitine is present to maintain high serum levels to increase triglyceride utilization and counterbalance any adverse metabolic effects of the triglycerides. Carnitine administration may be, therefore, started by intravenous perfusion and then continued by the oral or parenteral route.

As known, carnitine contains an asymmetric carbon atom and consequently exists in two stereoisomers. Either the racemate or the isolated isomers can be conveniently used in the method of the present invention, although it appears that the L-isomer is more active, while the D-isomer is slightly more toxic. Thus, the LD_{50} in rats and mice assessed for various routes of administration according to the Litchfield and Wilcoxon method is as shown in the following Table A. (Litchfield, J. T., and Wilcoxon, F., J. Pharm. Exptl. Therap. 96, 99. 1949).

TABLE A

product	animal	route	LD_{50} (mg/kg)
D,L-carnitine	rat	i.v.	995
D-carnitine	"	sc	10,000
D,L-carnitine	mouse	i.v.	610
D,L-carnitine	"	sc	6,000
D-carnitine	"	sc	5,400
L-carnitine	"	sc	7,000

The dose of acyl-carnitine which is administered will be determined by the attending physician having regard to the age, weight and condition of the patient, using sound professional judgement. Although effective utilization of exogenous glycerides can be noticed at doses as low as from 30 to 50 mg/kg of body weight daily, a dose of from about 150 to about 200 mg/kg of body weight daily is preferred. Should it be deemed necessary, larger doses can be safely administered, because of the extremely low toxicity of acyl-carnitine.

Some clinical studies are briefly summarized herein-below.

CASE 1

A 56 year old female patient was operated for removal of suppurated echinococcal cyst of the right lobe of the liver; after ten days of total parenteral nutrition initiated due to the severe sepsis exhibited by the patient: infusion was given via the central venous route of hypertonic glucose solution and a solution of amino acids and electrolytes for a total of 4000 ml daily with a 1:120 daily ratio of nitrogen: calories; via the peripheral route 500 ml of lipid emulsion (10%) containing acetyl carnitine 1.4% were infused every other day. Therapy was continued up to the 15th day after surgery and 4 days before the end normal cutaneous temperature was present and the patient had already begun to receive food through the mouth.

The following blood-composition parameters were checked on alternate days: glucose, BUN, Na^+, K^+, Ca^+, protides, albumin, transaminase, alkaline phosphatase, total bilirubin, total lipids, total and esterified cholesterol, triglycerides, haemochromocytometric test. With the exception of glycaemia, maintaining values between 120 and 200 mg%ml, bilirubin between 2 and 4 mg%ml with 1.2 mg%ml on the 15th day and alkaline phosphatase constantly showing values around 150 U/ml also upon discharge (normal up to 80 U/ml), the other parameters exhibited values within the normal range. The following were determined 8 times on different during parenteral nutrition: cardiac output using the Fick method, total peripheral resistances, artherovenous O_2 difference, O_2 consumption.

These assessments showed constant presence of cardiovascular hyperdynamism (cardiac output constantly between 3.1 and 4.8 l/min/mg with O_2 consumption between 140 and 230 ml/min/mg) demonstrating the effectiveness and good tolerance of the hypercaloric nutritional therapy.

CASE 2

A 50 year old male patient was hospitalized with cancer of cardia in malnutritional conditions; for approximately 4 months he had been complaining of worsening dysphagia for solids and liquids with an almost total impossibility to feed himself during the last 20 days. The patient, weighing 50 kg upon admittance, was given total parenteral nutrition by means of central venous catheter positioned for sub-clavian artery puncture. Daily administration: 1000 ml of glucose solution 40%, 1000 ml of an amino-acid solution 8.5%, 500 ml of lipid emulsion 5% containing acetyl carnitine 0.7%, vitamins and electrolytes. The parenteral nutrition was clinically well tolerated without giving rise to abnormal blood values. The blood parameters were checked every other day. In particular nitrogen and creatinine, total lipid, triglyceride and carnitine blood values remained within normal values. Now and then the ketone body urine value was positive using the ketostix test carried out every 12 hours.

After 15 days of nutrition the patient was in good conditions for surgery and his body weight had increased by 8 kg.

CASE 3

A 40-year old male patient was hospitalized because of a severe starvation caused by "short bowel syndrome"; seven months earlier, he underwent a large resection of the small bowel after mesenteric vein occlusion of unknown origin. Since then, he kept on feeding by mouth, sometimes trying hypercaloric diets enriched with median-chain-triglycerides or desultory cycles of hypocaloric parenteral nutrition; he seemed not to benefit by this treatment and he went on losing weight.

By the time he came under our observation, he weighed 44 kg and he had 4–5 bowel movements each day, so that total daily volume of stools was approximately 5 liters.

Total serum albumin was very low (2.2 g%ml); serum potassium was low (2.7 mEq/l); alkaline phosphatases and serum transaminases were slightly increased; anaemia was not severe (Hb 10 g%ml), but serum iron levels were quite low. He soon began total parenteral nutrition through central venous catheter and he was put on n.p.o.

Over a period of 4–5 days daily administration gradually reached 600 to 700 g of glucose, 100 g of amino acids, 500 ml of 5% lipid solution containing 0.7% acetyl-carnitine (peripheral route), vitamins, minerals and electrolytes; the daily amount of water given through the various routes was somewhere between 3000 to 4000 ml.

Serum potassium levels returned to normal only after 30 days of administering 200 mEq of potassium per day. Diarrhoea receded after 12 days of this treatment and only little amounts of mucus were passed with the stools. After 25 days of treatment the central venous catheter was removed because of systemic sepsis, and lipids and amino acids were administered for five days via the peripheral route, while glucose was interrupted.

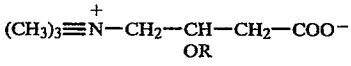
During this period, no particular alterations of serum values were observed: total lipids, serum triglycerides, total cholesterol and esters were always within the normal range. Parenteral nutrition by central venous catheter was begun again, though lipid 5% administration was changed to 500 ml of a 20% lipid solution with 2.1% acetyl-carnitine, twice weekly. For a further two months, parenteral nutrition was continued according to this schedule: minor changes involved electrolytes, glucose, and addition of insulin or albumin; the antral vanous catheter was replaced twice.

After three months of total parenteral nutrition, our patient weighed 54 kg; parenteral nutrition was interruptedn and he was fed on elementary diet. Though he continued to receive lipid administration for an addi-

tional week by the central venous route. One month after the beginning of this treatment, our patient had 2–3 bowel movements each day (nearly 1.5 liters on the whole) and body weight remained unchanged. He was discharged from hospital while on oral feeding (varied diet including few solid foods and precooked meat).

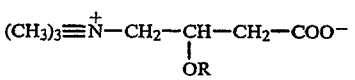
What is claimed is:

1. In the method of increasing in a human the level of fatty acids selected from the group consisting of oleic acid, linoleic acid, palmitic acid, stearic acid and linolenic acid by parenteral intravenous administration of one or more triglycerides of said fatty acid to a human, the improvement which comprises sumultaneously administering a quantity of an acyl-carnitine sufficient to enhance the oxidation of said fatty acids in the body of said human, said acyl-carnitine having the general formula



wherein R represents acetyl, propionyl, butyryl, hydroxy butyryl, hexanoyl, octanoyl, decanoyl, palmitoyl, stearoyl, acetoacetyl, succinyl, isovaleryl and crotonyl.

2. A method of treating patients in need of total parenteral nutrition, which comprises parenterally administering to a patient in need thereof an amount of physiologically acceptable triglycerides therapeutically effective for nourishing said patient and in combination therewith an amount of an acyl-carnitine of the general formula:



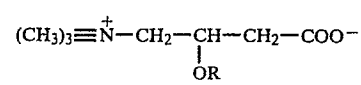
(I)

wherein R represents acetyl, propionyl, butyryl, hydroxy butyryl, hexanoyl, octanoyl, decanoyl, palmitoyl, stearoyl, acetoacetyl, succinyl, isovaleryl and crotonyl or a pharmaceutically acceptable salt thereof sufficient to increase free fatty acid oxidation.

3. The method according to claim 1, wherein said acyl-carnitine is an L-acyl-carnitine.

4. The method according to claim 1, which further comprises orally or parenterally administering daily for 12 to 24 hours after discontinuation of said triglyceride administration, from about 30 mg to about 200 mg of said acyl-carnitine per kg of body weight.

5. A parenterally administerable aqueous intravenous pharmaceutical composition for increasing in the body of a human recipient thereof the level of fatty acids selected from the group consisting of oleic acid, linoleic acid, palmitic acid, stearic acid and linolenic acid, said composition comprising a quantity of one or more triglycerides of said fatty acids sufficient upon hydrolysis in the body to afford a nutritionally effective amount of said fatty acid, and an amount of an acyl-carnitine or a pharmaceutically acceptable salt thereof sufficient to enhance the oxidation of said fatty acids in the body, said acyl-carnitine having the general formula



(I)

wherein R represents acetyl, propionyl, butyryl, hydroxy butyryl, hexanoyl, octanoyl, decanoyl, palmitoyl, stearoyl, acetoacetyl, succinyl, isovaleryl and crotony.

6. The composition according to claim 5, wherein said amount of acyl-carnitine or pharmaceutically acceptable salt thereof is from about 2 to about 30 g/l.

7. The composition according to claim 5 comprising:

soybean oil	50–200 g/l
glycerine	22–26 g/l
egg yolk phospolipids (ovolecithin)	10.5–12.5 g/l
acetyl-carnitine or a pharmaceutically acceptable salt thereof	5–25 g/l
sodium hydroxide	sufficient to adjust pH of composition to 5.5–9.0
distilled water	balance to 1 liter.

8. The composition according to claim 5, comprising:

cottonseed oil	100–200 g/l
soybean lecithin	10–30 g/l
sorbitol	40–60 g/l
D,L-α-tocopherol	0.5–1 g/l
acetyl-carnitine or pharmaceutically acceptable salt thereof	5–25 g/l
distilled water	balance to 1 liter.

9. The composition according to claim 5, comprising

sesame oil	100–200 g/l
glycerine	20–30 g/l
cetylstearylsulfonic acid	1–1.5 g/l
propionyl-carnitine or pharmaceutically acceptable salt thereof	3–20 g/l
distilled water	balance to 1 liter

10. The composition according to claim 5, comprising:

safflower oil	100–200 g/l
sorbitol	50–70 g/l
polysorbate	8–12 g/l
D,L-α-tocopherol	0.5–1 g/l
acetoacetyl-carnitine or pharmaceutically acceptable salt thereof	2–20 g/l
distilled water	balance to 1 liter

11. The composition according to claim 5, wherein said acyl-carnitine is L-acyl-carnitine.

12. The composition according to claim 7, wherein said acetyl-carnitine is L-acetyl-carnitine.

13. The composition according to claim 8, wherein said acetyl-carnitine is L-acetyl-carnitine.

14. The composition according to claim 2, wherein said propionyl-carnitine is L-propionyl-carnitine.

* * * * *

EXHIBIT 2

US005651980A

United States Patent [19]

Lanza et al.

[11] Patent Number: 5,651,980

[45] Date of Patent: Jul. 29, 1997

[54] **METHODS OF USE OF UNCOATED GEL PARTICLES**

[75] Inventors: **Robert P. Lanza**, Natick; **Willem M. Kühtreiber**, Shewsbury; **William L. Chick**, Wellesley, all of Mass.

[73] Assignee: **Biohybrid Technologies, Inc.**, Shrewsbury, Mass.

[21] Appl. No.: **228,134**

[22] Filed: **Apr. 15, 1994**

[51] **Int. Cl.**[6] **C12N 11/04**; A61K 9/52

[52] **U.S. Cl.** **424/424**; 424/422; 424/423; 435/174; 435/177; 435/243; 435/382; 514/866; 514/885; 514/907; 514/953

[58] **Field of Search** 435/174, 177, 435/240.22, 240.45, 243; 264/4.3; 424/422, 423, 424, 489; 514/866, 901

[56] **References Cited**

U.S. PATENT DOCUMENTS

3,615,024	10/1971	Michaels	210/490
4,322,311	3/1982	Lim et al.	252/316
4,324,683	4/1982	Lim et al.	252/316
4,352,883	10/1982	Lim	435/178
4,353,888	10/1982	Sefton	424/25
4,391,909	7/1983	Lim	435/78
4,409,331	10/1983	Lim	435/178
4,495,288	1/1985	Jarvis, Jr. et al.	435/241
4,663,286	5/1987	Tsang et al.	435/178
4,673,566	6/1987	Goosen et al.	424/19
4,689,293	8/1987	Goosen et al.	435/1
4,690,682	9/1987	Lim	604/891
4,744,933	5/1988	Rha et al.	264/4.3
4,822,534	4/1989	Lencki et al.	264/4.3
4,902,295	2/1990	Walthal et al.	623/11
5,084,350	1/1992	Chang et al.	428/402.2
5,227,298	7/1993	Weber et al.	435/178
5,260,002	11/1993	Wang	264/4.1

FOREIGN PATENT DOCUMENTS

WO93/03901 3/1993 WIPO .

OTHER PUBLICATIONS

Aebischer et al., *Biomaterials*, 12:50–56 (1991).

Iwata et al., *Diabetes*, 38:224–225 (1989).

Iwata et al., *Transplantation Proceedings*, 24:934 (1992).

Iwata et al., *Transplantation Proceedings*, 24:952 (1992).

Lanza et al., *Proc. Natl. Acad. Sci. USA*, 88:11100–11104 (1991).

Lanza et al., *Diabetes*, 41:1503–1510 (1992).

Lavoie et al., *Cell Transplantation*, 2:163–173 (1993).

Soon-Shiong et al., *Transplantation Proceedings*, 23:758–759 (1991).

Soon-Shiong et al., *Transplantation*, 54:769–774 (1992).

Soon-Shiong et al., *Proc. Natl. Acad. Sci. USA*, 90:5843–5847 (1993).

Sugamori et al., *Trans. Am. Soc. Artif. Intern. Organs*, 35:791–799 (1989).

Tanaka et al., *Biotechnology and Bioengineering*, 26:53–58 (1984).

Uludag et al., *Cell Transplantation*, 2:175–182 (1993).

Weber et al., *Transplantation*, 49:396–404 (1990).

Wiegand et al., *Transplantation*, 56:1206–1212 (1993).

Horcher et al., "Biocompatibility of Microbeads From Purified Alginates in Lewis– and BB–Rats," *Diabetologia*, 36(Supp1):Abstract 274 (Aug. 1993).

Zekron et al., "Prolonged Graft Function After Transplantation of Microencapsulated Islets Into Presensitized Recipients," *Diabetologia*, 36(Supp1):Abstract 721 (Aug. 1993).

Primary Examiner—John Pak
Assistant Examiner—Brian G. Bembenick
Attorney, Agent, or Firm—Louis Myers, Esq.; Lahive & Cockfield

[57] **ABSTRACT**

The invention covers a method of implanting a living donor cell into a host animal without inflammatory response or rejection of the donor cell by the host animal, by obtaining an uncoated particle of a biocompatible, temperature-independent gel that encapsulates the living donor cell, wherein the uncoated particle provides a molecular weight cutoff that prevents host animal immune cells from entering the particle, yet does not have to prevent entry of host animal IgG and complement into the particle, and implanting the uncoated particle into the host animal.

64 Claims, 6 Drawing Sheets

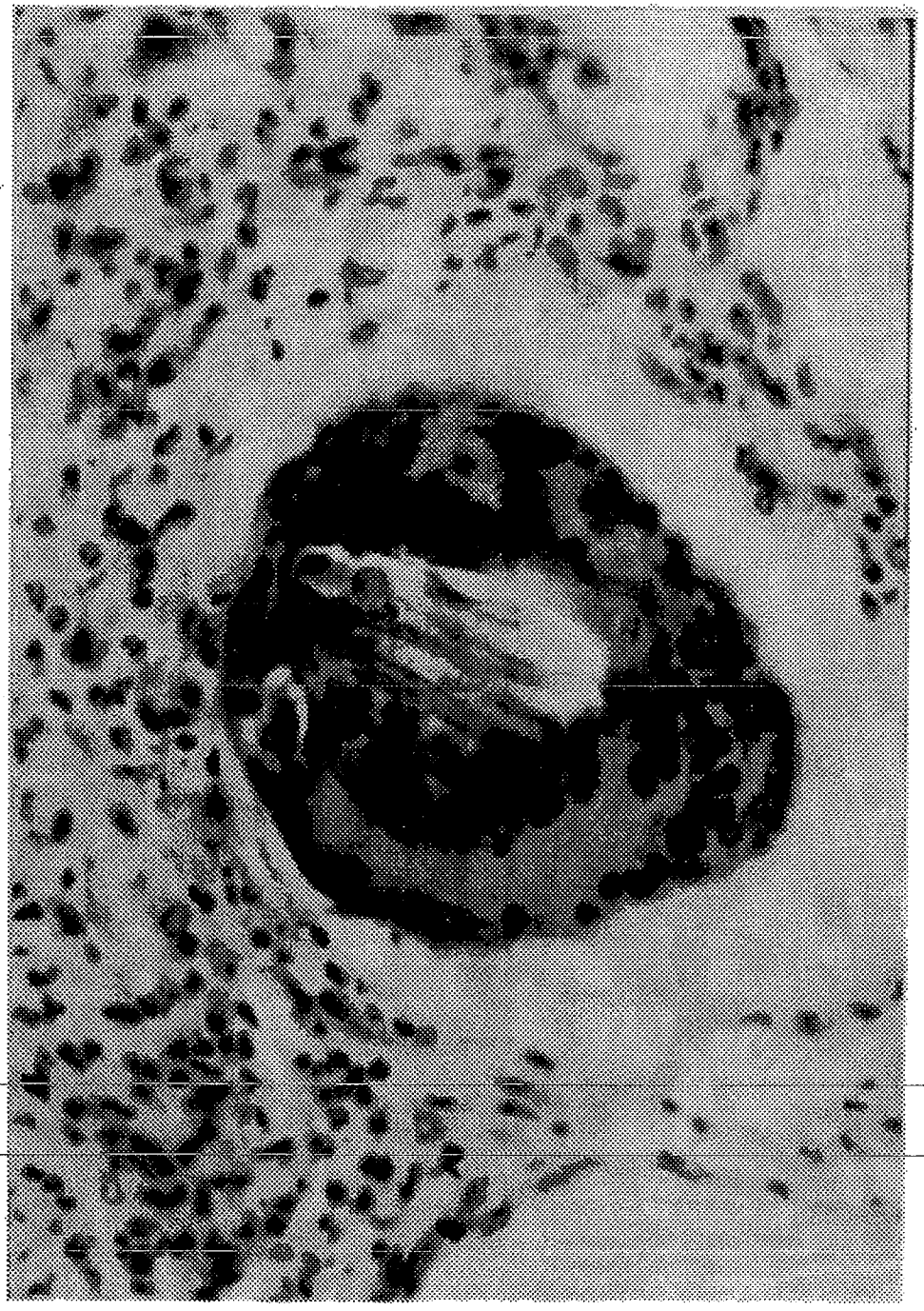


FIG. 1

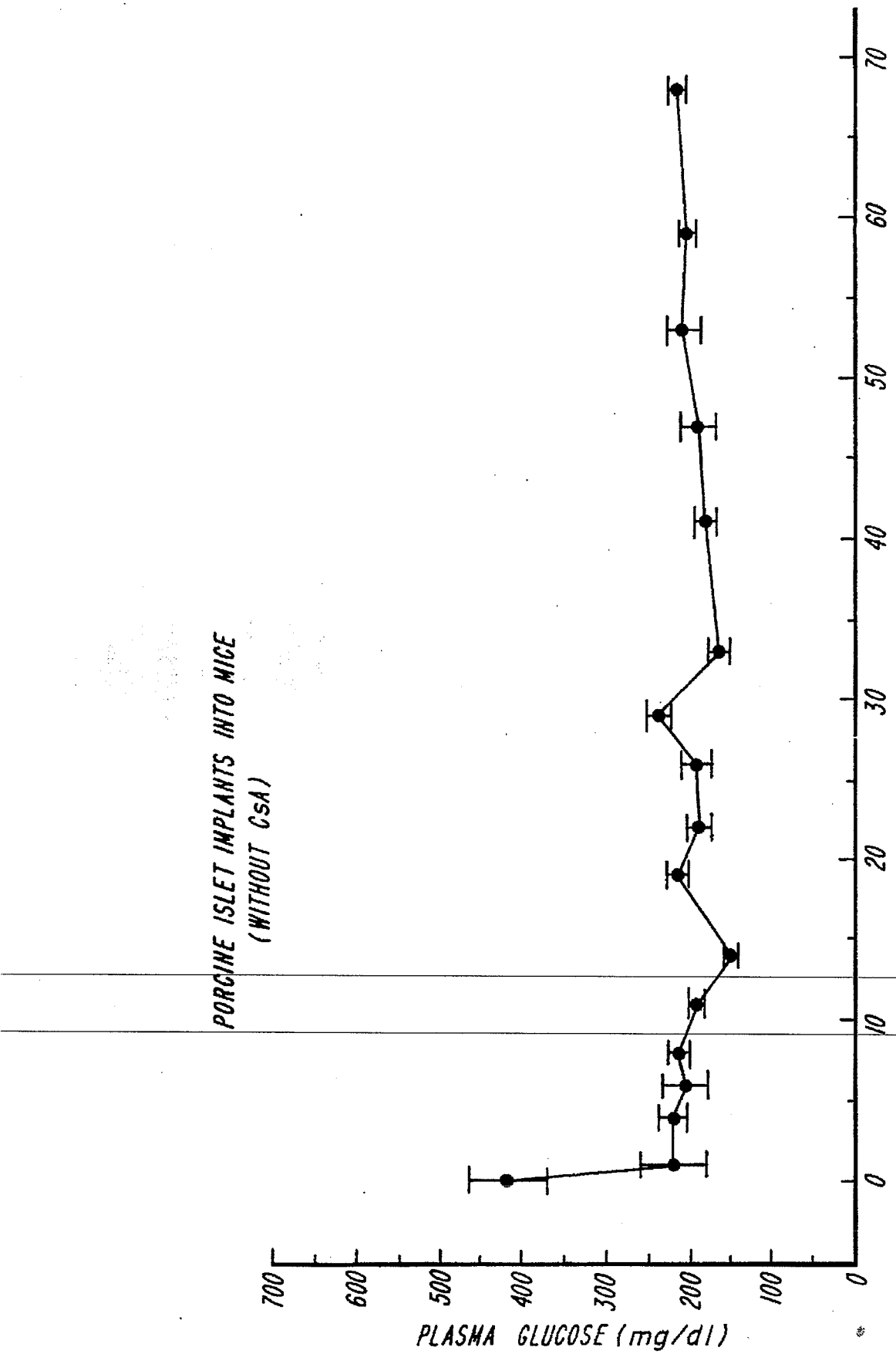

PORCINE ISLET IMPLANTS INTO MICE
(WITHOUT CsA)
PLASMA GLUCOSE (mg/dl)
700
600
500
400
300
200
100
0
0
10
20
30
40
50
60
70
DAYS POSTIMPLANTATION

FIG. 2

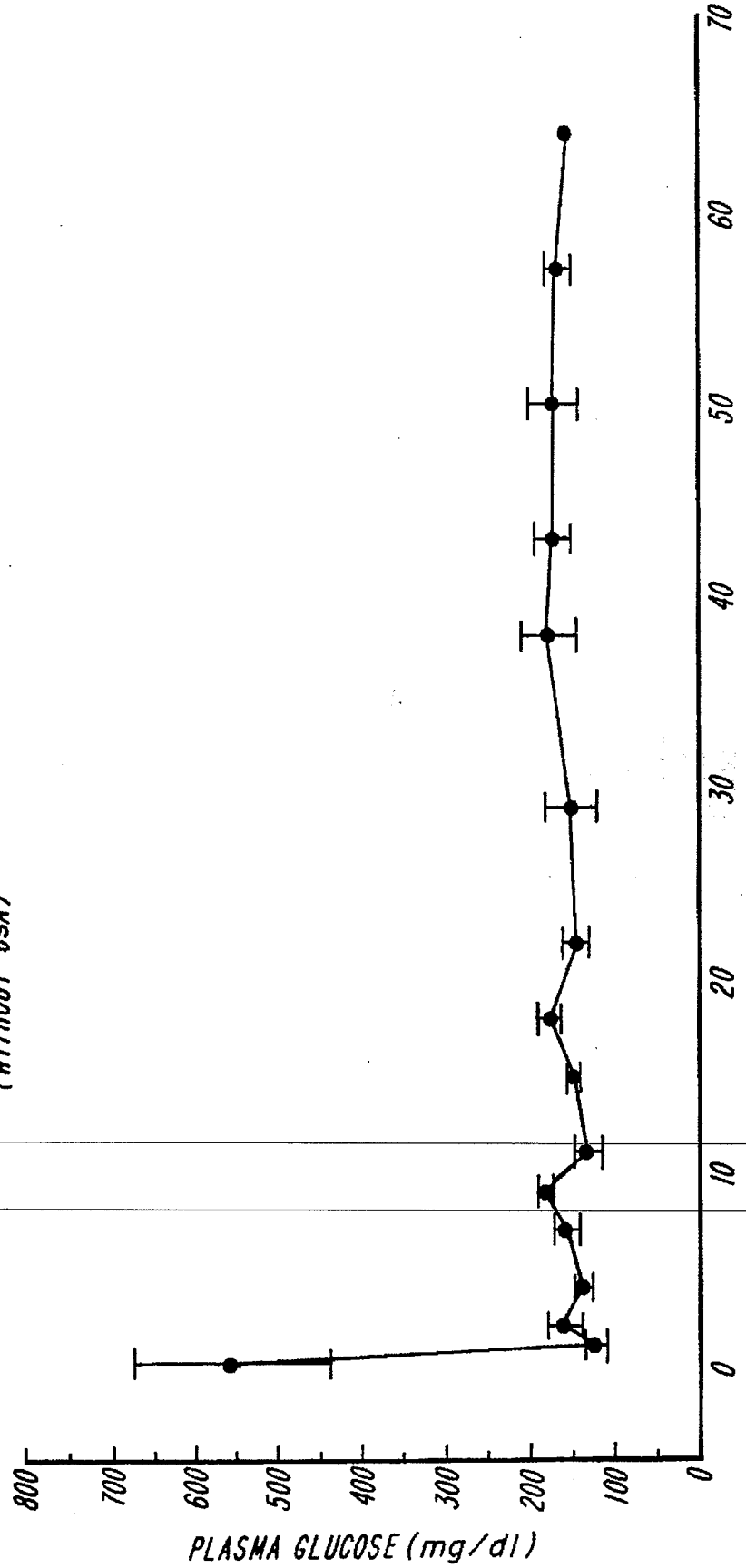

BOVINE ISLET IMPLANTS INTO MICE
(WITHOUT CsA)
PLASMA GLUCOSE (mg/dl)
800
700
600
500
400
300
200
100
0
0
10
20
30
40
50
60
70
DAYS POSTIMPLANTATION

FIG. 3

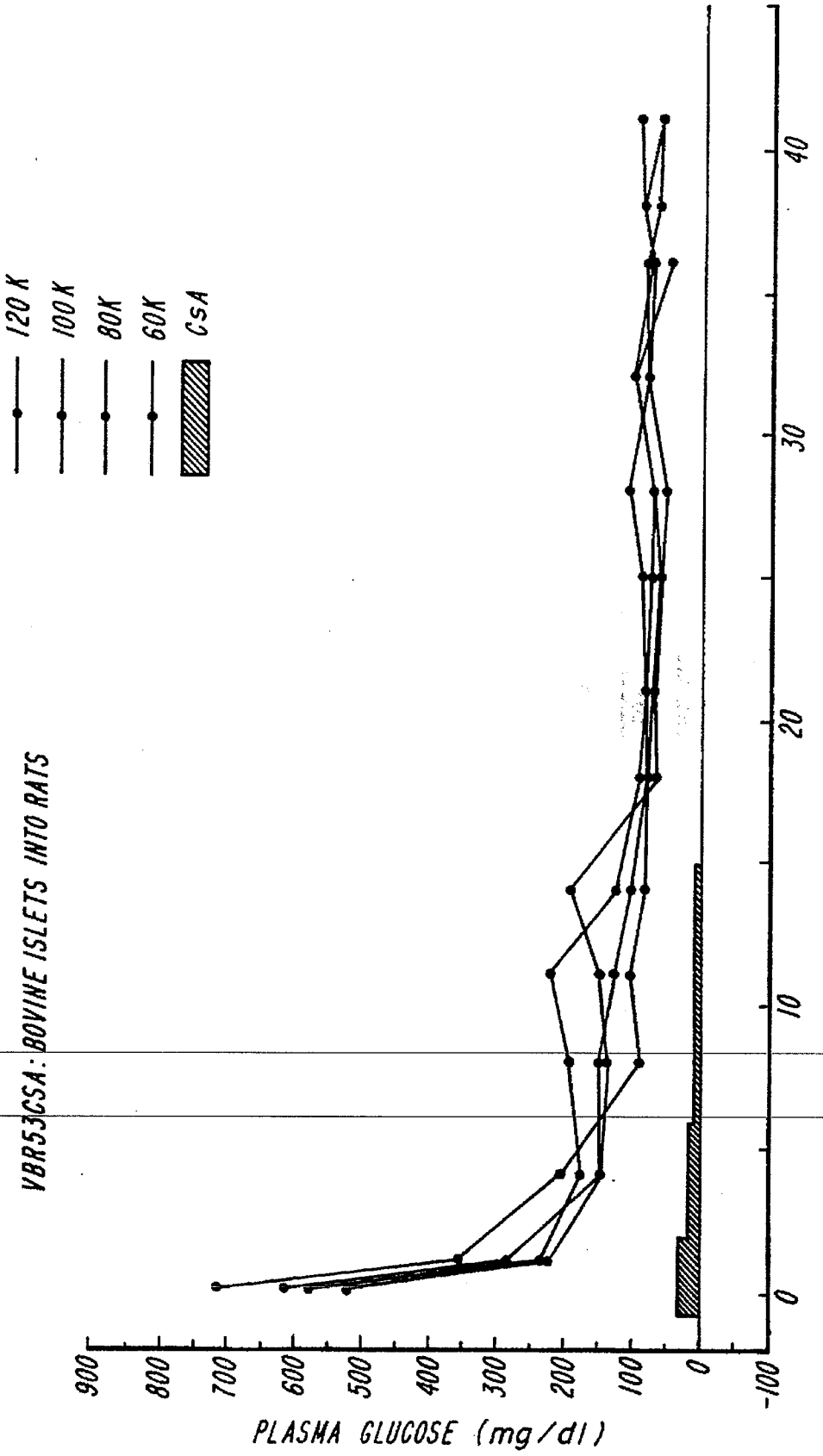

VBR53CSA: BOVINE ISLETS INTO RATS
120 K
100 K
80K
60K
CsA
PLASMA GLUCOSE (mg/dl)
900
800
700
600
500
400
300
200
100
0
-100
0
10
20
30
40
DAYS POSTIMPLANTATION

FIG. 4

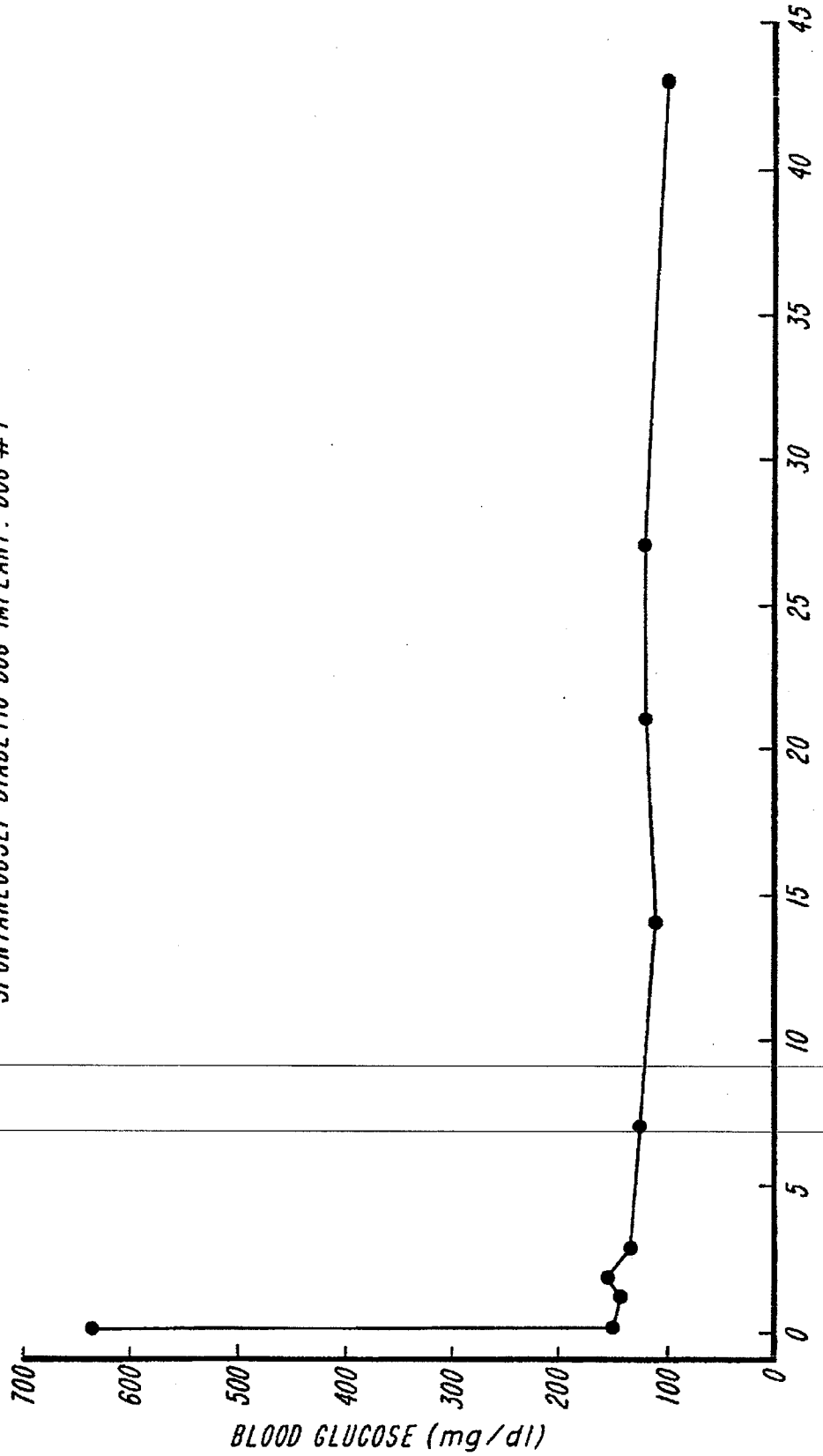

SPONTANEOUSLY DIABETIC DOG IMPLANT: DOG # 1
BLOOD GLUCOSE (mg/dl)
700
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
40
45
DAYS POST - IMPLANTATION

FIG. 5A

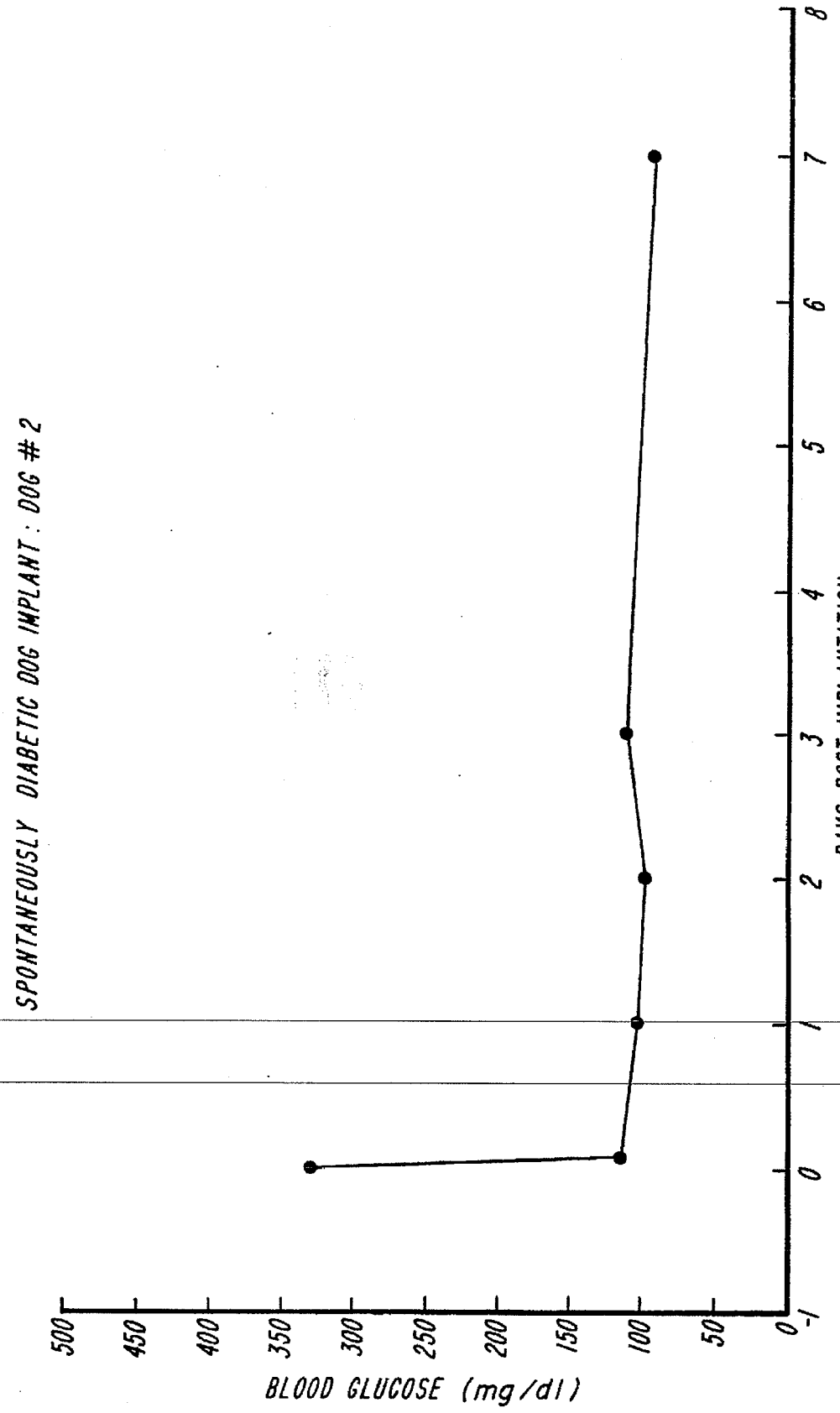

SPONTANEOUSLY DIABETIC DOG IMPLANT : DOG # 2
BLOOD GLUCOSE (mg/dl)
500
450
400
350
300
250
200
150
100
50
0
-1
1
2
3
4
5
6
7
8
DAYS POST IMPLANTATION

FIG. 5B

METHODS OF USE OF UNCOATED GEL PARTICLES

BACKGROUND OF THE INVENTION

The invention relates to methods of use of gel particles such as beads and spheres.

Gel microcapsules, e.g., of alginate, that contain a relatively small number of living cells have been used to transplant donor cells into host animals in both allografts, i.e., same-species transplants, and xenografts, i.e., different-species transplants. The microcapsules are used primarily in an attempt to immunoisolate the donor cells from the host's immune system. In the case of alginate microcapsules, they include an inner gel core and an outer semipermeable membrane or other coating with a controlled porosity to prevent components of the host's immune system from entering and destroying the cells within the microcapsule cores.

Several methods for microencapsulating cells, e.g., pancreatic islet cells, in alginate gels have been investigated. These include the alginate-polylysine technique described in Lim et al., U.S. Pat. No. 4,391,909 and Soon-Shiong et al., *Transplantation,* 54:769–774 (1992), the alginate-chitosan system described in Rha et al., U.S. Pat. No. 4,744,933, and the polyacrylate encapsulation method described in Sefton, U.S. Pat. No. 4,353,888. Each of these methods results in alginate gel microcapsules with an outer coating that is distinct from the inner core.

The alginate-polylysine technique, involves extruding a mixture of cells and sodium alginate into a $CaCl_2$ solution using a droplet generation device to form temporary gelled droplets. These droplets are then coated with positively charged polylysine to form a semipermeable outer membrane or coating around the gelled droplets. Tests have shown that these microcapsules are unstable and produce an inflammatory and fibrotic response when implanted into the peritoneal cavity of animals. However, the addition of a third outer alginate layer over the polylysine membrane has improved the biocompatibility of the microcapsules, resulting in an increase in the duration of islet allograft function in diabetic rodents to more than a year, as described in O'Shea et al., Biochem. Biophys. Acta, 804:133–136 (1984).

Although the alginate-polylysine microcapsules have been shown to prolong the survival of cells in allografts and xenografts, these microcapsules have typically required adjunctive treatment with immunosuppressive agents such as cyclosporin ("CsA"). However, when used in therapeutic, i.e., immunosuppressant, dosages, these agents cause a host of serious side effects including infection, cancer, and renal toxicity. Thus, the use of immunosuppressive agents in therapeutic dosages is undesirable.

Nevertheless, immunosuppressive agents are still used. For example, Soon-Shiong et al., *Transplantation,* 54:769–774 (1992) and Soon-Shiong et al., P.N.A.S., USA, 90:5843–5847 (1993), describe the use of alginate-polylysine-alginate microcapsules for allografts of canine islets into diabetic dogs, both with continuous or temporary, e.g., 30 day, immunosuppression with CsA. Both sets of dogs remained independent of insulin for an average of over 100 days.

In another report, Soon-Shiong et al., First *Int'l Cong. Xenotrans.*, p. 22 (Minneapolis, MN 1991), describes the prolongation of discordant islet xenograft function in streptozotocin-induced diabetic rats by alginate-polylysine microencapsulation. Microencapsulated canine and human islets were implanted intraperitoneally in the rats and compared to nonencapsulated islet implants. Low dose CsA therapy was instituted in both groups for the duration of the study. Euglycemia was maintained for 43 to 123 days for canine islets, and 42 to 136 days for human islets. In contrast, nonencapsulated islets achieved euglycemia for less than 2 days.

However, there are a few reports of uses of microcapsules without immunosuppression. For example, Weber et al., *Transplantation,* 49:396–404 (1990), describes a discordant, e.g., from unrelated species, xenograft in which alginate-polylysine microcapsules containing canine islets functioned for an average of only 11.5~3 days in diabetic NOD mice. However, immunosuppressive treatment with anti-CD4 monoclonal antibody allowed the cells in some of the recipient mice to remain functional for an average of 83 days.

In addition, Iwata et al., *Diabetes,* 38 (Supp. 1):224–25 (1988), describes the use of pancreatic islet cells encapsulated in agarose gel microspheres in concordant xenografts, i.e., transplants from different, but closely related species, e.g., rodent-to-rodent transplants, such as hamster cells into mice. No immunosuppressive agent was used in this study, and the two mice remained normoglycemic for 29 and 53 days, respectively.

In a second similar concordant xenograft study, Iwata et al., *Transplantation Proc.,* 24:952 (1992), the immunosuppressive effect of the drug 15-deoxyspergualin on host mice was compared with control mice that received no immunosuppression. Iwata et al. concluded that the agarose microspheres without immunosuppression could not effectively protect the concordant xenografts from rejection, because blood glucose levels indicated that only 2 of 8 xenografts survived over 100 days. However, blood glucose levels indicated that 3 of 5 xenografts survived over 100 days in mice receiving the immunosuppressive drug for 120 days (2.5 mg/kg/day) or 40 days (5.0 mg/kg/day).

In another study, Iwata et al., Transplantation proc., 24:934 (1992), used mouse islet allografts in agarose microspheres to achieve normoglycemia in diabetic mice without immunosuppression. Blood glucose levels indicated that the majority of these allografts survived over 100 days.

SUMMARY OF THE INVENTION

The present methods of using biocompatible, temperature-independent gel particles, e.g., beads, are based on the discovery that donor cells, e.g., porcine, bovine, or canine islet cells, encapsulated in alginate beads can be successfully transplanted into a host animal, e.g., mouse, rat, or dog, without any protective coating or semipermeable membrane around the beads, and with the use of only minimal doses, if any, of immunosuppressive or anti-inflammatory drugs. These simple, uncoated beads can be implanted into the host and provide effective immunoisolation of the encapsulated cells without eliciting a fibrotic response or a host immune rejection of the donor cells within the beads.

In general, the invention features a method of implanting a living donor cell into a host animal without inflammatory response or rejection of the donor cell by the host animal by obtaining an uncoated particle consisting essentially of a biocompatible, temperature-independent gel that encapsulates the living donor cell, wherein the uncoated particle provides a molecular weight cutoff that prevents host animal immune cells from entering the particle, and does not prevent entry of host animal IgG and complement into the particle, and implanting the uncoated particle into the host animal.

As used herein, a "temperature-independent gel" is a gel that can be gelled or crosslinked, e.g., by the addition of ions such as calcium, potassium, or barium ions, without a change in temperature. An "uncoated particle" refers to a bead, sphere, or other gel structure, e.g., a cylinder, that is composed of a biocompatible, temperature-independent gel matrix without any surface or intermediate layer, e.g., in the form of a semipermeable membrane, of a permeability and molecular weight cutoff different from that of the gel matrix itself.

As used herein, "molecular weight cutoff" refers to the size of the largest molecule that is not substantially blocked by a semipermeable membrane surrounding a microcapsule or by the gel matrix itself in an uncoated gel particle, e.g., bead, according to the invention. Molecules with a molecular weight above the cutoff are substantially prevented from entering or leaving the microcapsule or gel particle. The coatings of prior art alginate microcapsules generally provide a molecular weight cutoff of greater than 50,000 and less than 100,000 daltons. The uncoated gel particles of the invention have a molecular weight cutoff of greater than about 500,000 daltons, i.e., molecules like IgG and complement can enter these gel particles, but host cells such as immunocytes are prevented from entering these gel particles. In addition, this high molecular weight cutoff allows molecules secreted by the encapsulated cells, e.g., Factor VIII or hormones, to exit the gel particles.

The invention also features a method of implanting a living donor cell into a host animal without inflammatory response or rejection of the donor cell by the host animal by suspending the living donor cell in a liquid medium, the medium consisting essentially of water and a biocompatible, temperature-independent liquid gel, forming a droplet of the liquid medium that contains at least one living cell, solidifying the droplet to form a gel particle that encapsulates the living cell, whereby no outer coating is formed on the particle, and wherein the uncoated particle provides a molecular weight cutoff that prevents host animal immune cells from entering the particle, and does not prevent entry of host animal IgG and complement into the particle, and implanting the uncoated particle into the host.

In particular embodiments, when the liquid medium contains pancreatic islets, they can be present at a density of about 2 to 60 islets per mm^3, and more preferably at a density of about 10 to 35 islets per mm^3 i.e 10,000 to 35,000 islets per milliliter of the medium. When the liquid medium contains other living cells, they can be present at a density of about 10^5 to 10^8 cells, and preferably 10^6 to 10^7 cells, per milliliter of the medium. The density depends on the size and metabolism of the individual islets.

Furthermore, the invention features a method of treating a disease in a patient caused by a deficient production of a substance in the patient by obtaining an uncoated particle consisting essentially of a biocompatible, temperature-independent gel that encapsulates a living donor cell that secretes the substance, wherein the uncoated particle provides a molecular weight cutoff that prevents patient immune cells from entering the particle, and does not prevent entry of patient IgG and complement into the particle, and implanting the uncoated particle into the patient in a location and in a manner that allows the living cell to remain physiologically active and secrete the substance into the patient to treat the disease. For example, the uncoated particles can be implanted into an immunoprivileged site in the patient.

In particular embodiments, the disease is diabetes and the donor cell is a pancreatic islet cell. The donor cell can be selected to secrete Factor IX, Factor VIII, an interleukin, an interferon, an endocrine hormone, a nerve growth factor, tumor necrosis factor alpha, a neurotropic factor, or a neurotransmitter. The disease can be diabetes mellitus, hepatic disease, amyotrophic lateral sclerosis, hemophilia, hypothyroidism, Parkinson's disease, acquired immune deficiency syndrome, Duchenne's muscular dystrophy, infertility, epilepsy, Huntington's disease, hypoparathyroidism, a mood disorder, a motor neuron disease, osteoporosis, or Alzheimer's disease.

The invention also features an in vivo method of culturing a living cell by encapsulating the living cell in an uncoated particle consisting essentially of a biocompatible, temperature-independent gel, inserting the uncoated particle into an animal, and allowing the animal to thrive, thereby culturing the cell.

The invention further features, an in vitro method of culturing a living cell by encapsulating the living cell in an uncoated particle consisting essentially of a biocompatible, temperature-independent gel, placing the uncoated particle into a medium including nutrients and oxygen, and maintaining a sufficient amount of nutrients and oxygen in the medium to allow the cell to thrive, thereby culturing the cell.

In addition, the invention features a method of manufacturing uncoated, temperature-independent gel particles containing living cells consisting of the steps of suspending the living cells in a liquid medium, the medium consisting essentially of water and a biocompatible, temperature-independent, liquid gel, forming a droplet of the liquid medium, solidifying the droplet to form a gel particle that encapsulates the living cells, whereby no outer coating is formed on the particle, and storing the gelled uncoated particles in a nutrient medium to maintain the viability of the living cells.

In all of these methods, the living donor cell can be obtained from a species that is the same as or different from the host animal, and can be a genetically altered human cell. The host animal can be a dog or a human. The donor cell can be a porcine, bovine, canine, bacterial, fungal, or plant cell. In particular, the donor cell can be a pancreatic islet cell, or can secrete Factor IX, Factor VIII, an interleukin, an interferon, an endocrine hormone, a nerve growth factor, tumor necrosis factor alpha, a neurotropic factor, or a neurotransmitter.

In particular embodiments, the gel particle is spherical and has a diameter of from 50 to 6000 microns, and preferably from 2000 to 4500 microns. The gel can be an alginate or alginate derivative, and the alginate can be crosslinked with an ion, such as the calcium in a calcium salt. The uncoated gel particle can be biodegradable, and the rate of degradation of the gel in the uncoated particle can be selected to match the life expectancy of the donor cell.

In other embodiments, the uncoated particle encapsulates an autologous erythrocyte in addition to the donor cell, or can be treated with a nitric oxide inhibitor prior to implantation. In addition, the method can include the step of administering a drug to the host animal at a dosage effective to inhibit fibrosis and inflammation of the uncoated particle, but at a dosage lower than that required to achieve immunosuppression when the donor cell is implanted into the host animal without encapsulation. For example, the drug can be cyclosporin A and is administered at a dosage that achieves a whole blood trough level of less than about 100 ng/ml in the host animal. In addition, the drug can be administered for up to several weeks, e.g., one month, or longer, after implantation, and is then no longer administered.

The invention also provides a variety of other features which enhance implant function and longevity including size of the beads, types of gel matrices, and for islet cells, optimum cell densities. It is essential that the gel matrices keep host cells, i.e., immunocytes, physically separated from the donor tissue cells. It is also important that the particles have a sufficient size to keep antigens secreted by the encapsulated living cells from building up in the matrix and coming into direct contact with the host, and to protect the encapsulated donor cells from small soluble or cytotoxic factors in the host such as nitric oxide, lymphokines, cytokines, and natural killer (NK) cytotoxic factors. The charge and chemical properties of the matrix are also important in this respect.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a photomicrograph of a mouse macrophage digesting a 18 day old, fibrosed alginate gel bead (fibers in cell).

FIG. **2** is a graph showing the effect of porcine islet implants on plasma glucose level in mice.

FIG. **3** is a graph showing the effect of bovine islet implants on plasma glucose level in mice.

FIGS. **4** is a graph showing the effect of bovine islet implants on plasma glucose level in rats.

FIGS. **5**A and **5**B are graphs showing the blood glucose Level in diabetic patient dogs before and after implantation of canine islets with low dose cyclosporin.

DETAILED DESCRIPTION

Various types of donor cells can be isolated, encapsulated, and then implanted into a host according to the present invention.

Isolation of Cells

Cells are isolated from surrounding tissues or grown in culture by procedures known to the art, and are then suspended in a liquid medium prior to encapsulation. For example, pancreatic islet cells were prepared from either adult mongrel dogs, pigs, or bovine calves (0–2 weeks old) by a modification of the methods of Warnock and Rajotte, *Diabetes*, 37:467 (1988), as previously described in Lanza et al., *P.N.A.S. USA*, 88:11100–11104 (1991).

Briefly, aseptic, viable porcine pancreata were obtained under aseptic operating room procedures. After resection (warm ischemia for less than about 15 minutes), the glands were cannulated and infused with cold (4° C.) University of Wisconsin (UW) organ preservation solution. Pancreatic tissues were dissociated using an intraductal collagenase digestion procedure. The collagenase is delivered by peristaltic pump, and the digested pancreas is mechanically disrupted in a polypropylene dissociation chamber containing 2–6 mm glass beads. The islets were separated from the exocrine tissue by discontinuous density gradient centrifugation (27%, 20.5%, and 11% (w/v) FICOLL® (Sigma, F 9378) in Eurocollins solution).

Isolated islets were then cultured for one day either in M199/Earle's medium supplemented with 10% (vol/vol) fetal bovine serum, 30 mMHEPES, 100 mg/dl glucose, and 400 IU/ml penicillin (canine), or in α-MEMplus 10% heat-inactivated horse serum (bovine and porcine) in a humidified atmosphere of 5% CO_2/95% air at 37° C. A typical yield of islets should be in the range of $0.5–1.8\times10^6$ islets for adult pancreas (400 gmwet weight, islet diameter 80–125 μm, purity 85–95%, viability greater than 90% (see below). The cells may also be isolated by other procedures and cultured under other suitable conditions.

Ischemic deterioration of the islet cells is minimized by using tissue fragments of a suitable size, e.g., islet fragments should be less than about 120 microns, and preferably 40 to 100 microns, in diameter. Viability, growth, longevity, and/or function of the islet cells can be enhanced by co-culturing, i.e., by mixing other cell types in the liquid medium prior to encapsulation. Useful cell types include cells which secrete growth hormone, e.g., GH-3 cells, or cells which secrete connective tissue and/or extracellular matrix components, e.g., fibroblasts and endothelial cells. In addition, cells, e.g., islets, can be co-cultured with red blood cells, hemoglobin, or other oxygen carrying agents to enhance oxygen availability. Islet quality control procedures are used to enable comparison of different lots of islets prepared at different times. Purity (amount of islet tissue compared to exocrine tissue contamination) depends on the relatively unique characteristic of pancreatic islets to rapidly take up diphenyl thiocarbazone (dithizone). Islets are therefore incubated for five to ten minutes with 50 μg/ml of dithizone (D5130, Sigma) to stain them red. The preparation is then examined under light microscopy for a qualitative estimate of purity. Quantification of purity is effected by islet dispersion and counting of stained and unstained cells, or with a spectrophotometric assay of dithizone uptake/μg DNA.

Viability can be determined by any one of several assays that depend on the capability of viable cells to exclude certain dyes. For example, one assay uses a combination of the fluorescent stains acridine orange, which stains only viable cells green, and propidium iodide, which stains only the nuclei of dead cells red. The islets are incubated with the dyes (acridine orange, Sigma A6014, 50 μg/ml, and propidium iodide, Sigma P4170, 2.5 μg/ml) in a PBS solution for 10 to 15 minutes and then dispersed into single cells. Counts of red and green fluorescing cells are used to calculate % viability.

Insulin secretory activity of the islets is determined both in static culture, e.g., expressed as units of insulin per islet volume, and based on the capability of the islets to respond to graded concentrations of glucose. These values are quantitatively established by measuring the insulin secreted by islets exposed to a range of glucose concentrations extending from 2.8 to 28 mM glucose.

Encapsulation

Once the cells are isolated and suspended in liquid medium, they must be encapsulated by a supporting gel matrix. Beads suitable for implantation into a host animal include a number of living donor cells in a gel matrix without any protective coating. Using standard techniques, a gel matrix is formed by adding cells, e.g., pancreatic islets, to a solution of nutrient medium and liquified gel, e.g., sodium alginate, to form a suspension, and then crosslinking the gel, e.g., by adding a crosslinking agent such as calcium

chloride. The gel matrix can be any one or a combination of a variety of substances that are biocompatible with the host animal, and are capable of maintaining cellular viability and physically supporting the tissue or cells in suspension, as long as they have the required concentration and purity.

The gels must be temperature-independent, in that they can be gelled or crosslinked, e.g., by the addition of ions such as calcium, potassium, or barium ions, without a change in temperature, which could be harmful or fatal to the living cells to be encapsulated. Temperature-independent gels include alginates, carrageenans, and gums such as xanthan gum. As used herein, the term alginate includes alginate derivatives. These gels should be treated to remove polyphenols, lipopolysaccharides, endotoxins, and other impurities using standard techniques.

Alginate is composed of blocks of 1,4 linked β-D-mannuronic acid (M) and α-1-guluronic acid (G) linked together, e.g., in alternating MG blocks. The preferred alginate is one formulated with a high G block content, e.g., at least about 60 percent. The higher the percentage of G blocks, the greater the pore size and the strength of the gel matrix. In addition, it has been noted that alginate gels with a high M block content appear to be more immunogenic than gels with a high G block content. See, e.g., Soon-Shiong et al., *Transplant. Proc.*, 23:758–759 (1991), and Soon-Shiong et al., *Transplantation*, 54:769–774 (1992).

The gel matrix should be sufficiently viscous to maintain the cells in a dispersed state. When alginate is used as the gel matrix, it is added up to about 3%, preferably to about 1 to 2%, of the liquid medium, and the solution is cross-linked to form a semisolid gel in which the cells are suspended. These percentages provide a matrix that maintains its shape and has sufficient mechanical strength to remain intact in vivo for several months.

For example, pancreatic islets can be encapsulated as follows. After preculturing overnight, islet cells were suspended uniformly at a density of 20,000 islets/ml, which is 20 islets/mm^3, in a solution of 1.5% (wt/vol) Pronova LVG sodium alginate (Protan, Drammen, Norway) in culture medium plus additives (α-MEM, 10 mMHEPES pH 7.1, penicillin, 2 mM glutamine for porcine islets; and M199 with the same additives for canine islets). A syringe pump was used to pump the suspension through an air jet apparatus (containing a straight-edged 22 gauge needle) at a speed of 3 ml/min. Droplets formed at the tip of the needle were stripped off by means of a concentric flow of air at an air speed of 7 to 8 m/sec. The resulting droplets fell a distance of 4 cm and were collected in a solution of 1.5% $CaCl_2$ in 10 mMHEPES (pH 7.1) to form gelled beads. These beads can be made in various sizes ranging from about 700 μm to 3500 μm in diameter by altering the air flow speed, the faster the flow rate the smaller the beads.

Each bead contains approximately 1 to 25 islets. After three minutes, the beads were washed three times with culture medium (appropriate for the species of islets in use), and were then cultured in a tissue culture incubator at 37° C. and 5% CO_2 until they were implanted.

Larger beads up to 3500 to 6000 mm in diameter, were or can be made in a similar manner, or can be extruded through a syringe with a 14 gauge catheter. Beads can also be made by other standard techniques, as long as the resulting beads have the preferred characteristics described below.

The beads were cultured in vitro for up to four weeks, and the insulin secretion compared to free islets, prepared as described above. The insulin secretory response of the beads was approximately 50 to 80% of that of the free islets. Histological examination at four weeks revealed viable endocrine tissue within the beads. The islets were morphologically intact, and contained well granulated β-cells.

Specific Parameters for Uncoated Particles

The particles, e.g., beads, are preferably spherical in configuration and have a diameter of about 600 to 6000 μm, preferably 1500 to 3500 μm. Particles as small as 50 μm can be made. The preferred size is based on diffusion distances from the surface of the bead to the cells within. In addition, smaller beads, e.g., 700 to 900 μm are suitable for allographic transplants, whereas larger beads, e.g., 2000 to 5000 μm, are preferred for xenographic transplants.

A spherical shape is preferred for the beads to present a smooth outer surface without edges, which tends to inhibit fibrotic encapsulation of the beads. The beads may be designed to be more or less biodegradable depending on the intended use. For example, if the beads are intended to break down within a certain period of time, materials such as cellulose or collagen can be added to the gel matrix to facilitate the breakdown. However, as shown in FIG. 1, applicants have discovered that even pure alginate beads are attacked and digested by macrophages after the gel particle has been coated by fibrotic tissue by the host. FIG. 1 shows a macrophage with alginte fibers in the center of the cell. Other degradative mechanisms also occur. The resulting breakdown products are resorbed by the body, or excreted in the urine as segments of crosslinked or uncrosslinked alginate molecules. This breakdown of the beads may start within a few weeks or months, or within a year, and is controlled by the size of the beads, the crosslinking agent used to form the beads, and the added ingredients such as collagen, which dissolves on its own to weaken the bead structure after a few weeks. In general, this breakdown typically occurs after 6 to 12 months.

Other characteristics of the uncoated beads include (1) morphological and chemical properties, e.g., the smoothness of surface, the structure of the matrix, and the ability to react with other chemical substances, and (2) transport properties, e.g., permeability to microsolutes, nutrients, O_2, wastes, macrosolutes (e.g., insulin), essential proteins, and molecular weight cutoff to prevent immune cells (lymphocytes/macrophages) from entering the bead as discussed above. Both the morphological and transport characteristics are achieved by the gel matrix which physically isolates the donor cells from the host cells and allows nutrients and oxygen to flow freely into the matrix, which facilitates viability of cells. In addition, the negative charge of some gels, e.g., sodium alginate, should aid in preventing proteins of the humoral immune response (complement/cytokines) from entering the gel particles.

Donor Cell and Host Characteristics

The living donor cells are preferably mammalian cells, but can also be bacterial, fungal, or plant cells that express or secrete a desired protein, hormone, or other substance. The characteristics of these encapsulated donor cells are important to the survival of cells in the particles once implanted into a host. For example, the total antigenic load should be kept as low as possible while still implanting a sufficient number of donor cells to achieve the desired therapeutic effect.

This antigenic load can be controlled by adjusting the density of cells per bead and/or by adjusting the total number of beads implanted into the host. These numbers vary depending on the cell type and the type of host. For example, in a dog example described below, diabetes was treated with

beads made from 32.0 ml of gel containing porcine islets at a density of about 20 per mm3. To standardize islet dosages, the EIN (equivalent islet number) can be used. This number is based on the islet volume of a standard islet of 150 microns in diameter.

The total EIN implanted into a patient depends on the insulin requirements of the patient, and on the metabolism, type, and quality of the islets, which is determined by in vitro tests of the encapsulated islets prior to implantation as described herein. For example, it is known that porcine islets produce more insulin than bovine or canine islets. The amount of insulin (insulin units) required by a patient is determined empirically on an individual basis, and is based on sugar levels monitored several times per day. For example, diabetic dogs may require about 5 to 40 units of insulin per day, whereas a typical human diabetic patient may require 20 to 50 units per day. In all cases, these amounts depend on the severity of the disease, diet, exercise, and other factors. About 1.0 to 2.5 million porcine islets are required to achieve this level of insulin production for a human patient.

In addition, the immunogenicity of the donor cells must be considered. For example, it is believed that fetal or neonatal tissue will provoke less of a host reaction than adult tissue. The donor tissue can also be modulated to reduce its immunogenicity prior to implantation, e.g., by organ culture, UV irradiation, and/or pretreatment with antibodies to mask the antigens on the surface of the donor cells. Organ culturing selectively removes dendritic cells (antigen presenting cells) from the donor tissue since they die faster than other cells in culture. Culture conditions such as high oxygen and low temperature are effective to selectively destroy the more sensitive dendritic cells. All of these methods of modulating donor tissues are described in Chapters 9, 10, 11 of Lanza et al. (eds.), *Immunomodulation of Pancreatic Islets* (RG Landes, Tex., 1994), which is incorporated herein by reference.

The immune system of the host can also be modulated prior to or after implantation of the encapsulated donor cells to ensure survival of the implanted cells. Allografts in mammals larger than mice or rats require a short course of an immunosuppressant or anti-inflammatory drug at a low dosage. Discordant xenografts with small beads, e.g., 700 to 900 μm in diameter, in larger mammals such as humans also require adjunctive immunosuppression. As discussed below, minimal or no immunosuppression or anti-inflammatory therapy is necessary with larger beads.

Immunosuppressant drugs include cyclosporine A ("CsA"), FK-506, and deoxyspergualin. Anti-inflammatory/anti-fibrosis drugs include steroidal drugs such as prednisone, and non-steroidal drugs such as ibuprofen and aspirin. Certain immunosuppressants such as CsA have an anti-fibrosis effect at very low, "subtherapeutic" doses, e.g., at a so-called "whole blood trough level" of less than 100 ng/ml when analyzed by HPLC. Initial doses can be higher, e.g., up to a few hundred ng/ml, without attaining a therapeutic, e.g., immunosuppressive, dose, which is in the range of 550 to 900 ng/ml in dogs for unencapsulated xenogeneic islets. Thus, CsA can be used as an effective anti-fibrotic without the need for any other drugs. The maintenance blood levels of less than 100 ng/ml, e.g., 30 ng/ml, in allografts can be discontinued within several weeks to less than three months.

In human patients, the maximum therapeutic dosage of CsA should be less than 800 ng/ml to avoid toxicity problems. However, according to the invention, only low doses, e.g., an initial dosage of an immunosuppressive/anti-fibrotic agent of a few hundred ng/ml, and then a maintenance dosage of less than 100 ng/ml, should be administered.

In addition, according to in vitro observations, the living donor cells within the gel beads can be protected from cytotoxic nitric oxide radicals, by co-encapsulating the cells with autologous erythrocytes, which scavenge nitric oxide that may enter the gel beads once implanted, e.g., as described in Wiegand et al., *Transplantation,* 56:1206–1212 (November 1993). In addition, the gel beads can be treated with nitric oxide inhibitors such as N^G-methyl-L-arginine prior to implantation to provide a protective effect.

Implantation

The beads can be simply implanted into a host by injection with a standard catheter or syringe, e.g., with a 16 gauge needle for beads less than 1000 μm in diameter. Larger beads can be inserted via a small incision, e.g., with a catheter or funnel-like device. The beads are preferably implanted into the host intraperitoneally. The beads can also be implanted intramuscularly or subcutaneously. Alternatively, the beads can also be implanted into immunoprivileged sites such as the brain, testes, or thymus, where the host's immune response is least vigorous, as described in Chapter 7 of Lanza et al. (eds.), *Immunomodulation of Pancreatic Islets* (RG Landes, Texas, 1994). In addition, the beads can be inserted through a small surgically created opening using a gun/trocar type device that slips the beads under the skin.

EXAMPLES

Implantation of Porcine Pancreatic Islets into Mice and Rats

To determine whether encapsulated pancreatic islet cells can function, e.g., secrete insulin in a host animal, over extended periods of time, 800±100 μm diameter beads, containing a total of between 10K and 100K islets per animal, were implanted as discordant xenografts into mouse and rat diabetes mellitus models. Adult male Lewis rats (Charles River, Wilmington, MA) weighing 250 to 300 g, and C57BL/6J mice weighing about 20 to 30 g were used as implant hosts. Diabetes mellitus was induced in these animals by a single injection of streptozotocin ("STZ") ten to fourteen days prior to implantation of the beads. Rats were injected with 42 mg/kg body weight of STZ into the tail vein. Mice were injected with 165 mg/kg body weight of STZ into the peritoneal cavity.

Fasting plasma glucose concentrations (mg per dl) were measured by tail bleedings from both animals using a glucose oxidase method (Beckman Glucose Analyzer 2, Fullerton, CA). Determinations were performed thrice weekly for one month, and then weekly for the duration of each study. Failure of the encapsulated islets to reverse hyperglycemia was considered to have occurred when glucose concentrations exceeded 250 mg per dl on two consecutive testings.

Host animals were anesthetized with ketamine/xylazine (rats, 0.5 μl/g i.m.; mice, 5.0–7.5 μl/g i.p.) prior to implantation. The porcine islets were isolated and encapsulated in 800 μm diameter beads as described above. Between 10K and 100K islets were implanted into the peritoneal cavity of the rats or mice either with a 16 gauge catheter or through a small (1–2 cm) midline incision. This corresponds to a total of about 0.5 to 5.0 mls of gel which is formed into the beads, i.e., the islets are present in the gel at a density of about 20K islets/ml of gel. The wound was closed in two layers with 4–0 silk suture. No immunosuppressive drugs were used.

As discussed below, the beads generally reversed hyperglycemia in the hosts. This condition was confirmed by histological analysis. The encapsulated islets were recovered from streptozotocin-induced diabetic animals sacrificed two weeks after implantation and were routinely fixed and examined histologically. Donor islets were fixed in Bouin's solution, and then dehydrated and embedded in paraffin by routine histologic methods. The tissue was sectioned serially (5 μm sections) and stained with hematoxylin-eosin. The presence of insulin, glucagon, and somatostatin in donor islets was determined using immunoperoxidase histochemistry as described in Warnke et al., *J. Histochem. Cytochem,* 28:771 (1980) or Like et al., *Lab. Invest.;* 38:340 (1978). These tests are used to determine whether all these hormone-secreting cell types in the islets are viable.

Such histological tests are the only accurate method to determine the viability of islets after implantation in chemically induced diabetic animals, because it is common for such animals to revert to a non-diabetic state, which gives a false indication of islet viability if determined only by blood tests such as blood glucose levels. In addition, blood tests provide no indication of fibrosis.

As shown in FIG. **2**, porcine islets (approximately 20,000) encapsulated in 800±100 μm diameter beads made from 1.0 ml of alginate gel, and implanted into diabetic mice, reversed hyperglycemia for more than 10 weeks as evidenced by a drop in the plasma glucose level immediately after implantation from about 400 to 450 mg/dl to about 200 mg/dl, and the maintenance of the plasma glucose level at about 200 to 250 mg/dl.

These results were confirmed by histological analysis, which demonstrated intact, viable islets after 10 weeks. In addition, the beads showed little or no fibrosis for the 10 week period. Control experiments in which approximately 100,000 non-encapsulation canine, bovine, or porcine islets were implanted intraperitoneally into STZ-induced diabetic Lewis rats showed that these xenografts all failed within one week.

In other experiments, porcine islets were immobilized in five different sizes of uncoated alginate beads (880, 1600, 2200, 3000, and 3700 μm in diameter). These were implanted into the peritoneal cavity of STZ-induced diabetic rats for 11 to 14 days (n=2 for all tests). No immunosuppressant was used in these experiments. No islets survived in any of the 800 μm or 1600 μm diameter beads. Thus, it appears that smaller diameter beads containing porcine islets do not work without immunosuppression in rats. In other experiments, loss of blood glucose control and histology confirm that smaller diameter gel beads are rejected within about 6 to 10 days after implantation.

However, discordant xenograft studies using larger beads, e.g., 2200, 3000, and 3700 μm diameter beads, showed that porcine islet cells remained viable for more than four weeks in rats without any immunosuppression. The results (percent viability after 4 weeks) are shown in Table 1 below.

TABLE 1

Uncoated Bead Diameter (μm)	Percent Viability
800	0
1600	0
2200	33 to 44
3000	50 to 75
3700	80 to 85

Thus, larger diameter beads are successful in protecting the donor porcine islets from the host rats' immune systems without any immunosuppressant or anti-fibrotic drugs.

Implantation of Bovine Pancreatic Islets into Mice and Rats

In another discordant xenograft study, bovine calf pancreatic islet cells were isolated and encapsulated as described above for the porcine islets. Again, these beads were analyzed in the mouse and rat diabetes mellitus model described above.

As shown in FIG. **3**, bovine islets (approximately 20,000) encapsulated in 800±100 μm diameter beads made from 1.0 ml of alginate gel, and implanted into mice reversed hyperglycemia for greater than 60 days as evidenced by the immediate drop in plasma glucose level after implantation from about 550 mg/dl to about 150 mg/dl, and the maintenance of this level for the duration of the study. Histological analysis demonstrated intact, viable islets for more than 60 days, with little or no fibrosis.

Similarly, different numbers of encapsulated bovine islets were implanted into rats. CsA was administered subcutaneously to the rats for the first two weeks of the study and then discontinued (30 mg/kg on days −1, 0, and 1; 15 mg/kg on days 2 to 5; and 7 mg/kg on days 6 to 14). As shown in FIG. **4**, all four dosage levels of islets (60K, 80K, 100K, and 120K) reversed hyperglycemia for more than 40 days. Plasma glucose levels fell from about 525 to 725 mg/dl to less than 250 mg/dl after implantation, and maintained these levels for the duration of the study.

Histological analysis demonstrated intact, viable islets at 64 days, with little or no fibrosis.

Implantation of Islet Cells into Dogs

In an allograft study, donor canine islets (approximately 100,000) were encapsulated in 800±100 μm diameter beads made from 5.0 ml of alginate gel and implanted into dog hosts. Although such allografts are normally rejected by a host within 7 days, the encapsulated donor islets were all viable after three weeks. One dog received no immunosuppressant drug, and another received one daily injection of 10 mg/kg CsA, which provides a blood trough level of about 200 to 300 ng/ml, depending on the actual time of administration, liver function, and time of measurement. There was no fibrosis of the implanted beads in either dog, even without the use of any immunosuppressive agent in one of the dogs. Histology showed that 50% of the islets were viable in both dogs after three weeks. Thus, the allografts were successful with or without the use of immunosuppressant or anti-fibrotic drugs.

In a discordant xenograft dog study, porcine pancreatic islets (approximately 140,000) were isolated and encapsulated in 800±100 μm beads made from 7.0 ml of alginate gel and implanted as described above for the porcine islets. These beads were analyzed in a normal dog. The immunosuppressant CsA was administered orally to the dog at a dosage of 10 mg/kg/day throughout the course of the study. Histological analysis showed some viable islets after three weeks.

As a control, empty beads of the same size were injected into dogs to determine whether the beads themselves caused any inflammation or other immune reaction in the host. Histological examination showed that neither the empty nor the islet-containing beads had developed any fibrosis after over one month inside the dog host.

Treatment of Diabetic Dogs

In another dog study, two actual diabetic patient dogs were treated by implantation of about 600,00 to 650,000 canine islets in 800±100 μm beads made from 30.0 to 33.0 ml of alginate gel and implanted as described above. The first diabetic dog required about 11 to 12 units of insulin per

day prior to implantation. As shown in FIG. 5A these implanted beads reversed hyperglycemia for over six weeks as evidenced by the immediate reversal of hyperglycemia (drop in plasma glucose level after implantation from about 650 mg/dl to about 150 mg/dl), and the maintenance of the plasma glucose level at about 125 mg/dl for the duration of the study, without the need for any external administration of insulin.

In addition, at one month after implantation, a bolus intravenous injection of glucose caused only a transient rise in blood glucose level to 300 mg/dl, which was normalized within about 1 hour (data not shown). Prior to implantation, the same test showed a sugar level of over 600 mg/dl and remained significantly hyperglycemic for the duration of the test (greater than 600). These pre- and post-implantation tests provide evidence that the animals are truly diabetic absent the implants.

The second diabetic dog required about 8 to 10 units of insulin per day prior to implantation. As shown in FIG. 5B these implanted beads reversed hyperglycemia for over 7 days as evidenced by the immediate drop in plasma glucose level after implantation from about 350 mg/dl to about 100 mg/dl, and the maintenance of the plasma glucose level at about 100 mg/dl for the duration of the study, without the need for any external administration of insulin.

In this study, a low dose of CsA was administered to each dog. CsA was administered at a dosage of 10 mg/kg/day for the first two weeks, and then dropped to 5 mg/kg/day. However, by 21 days, HPLC analysis of the dog's blood showed no detectable trace of CsA. Thus, the blood level of CsA was less than about 30 ng/ml, which is the lowest detectable limit of this measurement technique.

Factor IX Expressing Cells

HeLa cells, primary rabbit fibroblast cells (WHHL), and hepatoma cells (HepG2) were engineered to overexpress human Factor IX by transfection with one of two human Factor IX expressing retroviral sequences, Moloney murine Leukemia virus LTR (Mo-LTR) or Myeloproliferative sarcoma virus LTR (MPSV-LTR). Human FIX is expressed from the retroviral 5' LTR promotor, while the dominant selectable marker npt (neomycin phosphotransferase; neo resistance; G418 resistance) is expressed from an internal promoter. Three of the four cell populations (one of each of HeLa, WHHL, and HepG2) were generated with the same retroviral vector which uses the Mo-LTR. The fourth population (WHHL) was generated using MPSV-LTR. All four cell populations secreted detectable levels of human FIX as determined by ELISA.

These cells were then encapsulated as follows. 1×10^6 or 2×10^6 cells were encapsulated in 800±100 μm diameter beads made from 1.0 ml of alginate. These beads were then cultured in vitro in DMEM in high glucose (G418 at a concentration of 0.8 mg/ml) to determine the amount of human Factor IX secreted by these encapsulated cells. The encapsulated cells produced high levels of Factor IX. Selection for G418 should be applied if the cells are grown for more than 2½ weeks in culture.

To determine the levels of Factor IX that these encapsulated cells produce in vivo, they are injected i.p. into mice. Untreated mice serve as controls. Plasma human Factor IX concentrations are measured at various time intervals after implantataion by tail bleedings using an enzyme immunoassay (Asserachrom IX:Ag; American Bioproducts).

Uses of Uncoated Gel Beads

The uncoated gel beads of the invention can be used to treat a variety of diseases that result from the defective or insufficient production of a particular enzyme or hormone by the body. In effect, the current methods provide a type of replacement therapy. A number of well-characterized disorders caused by the loss or malfunction of specific cells in the body are amenable to replacement therapy. For example, islets of Langerhans can be used for the treatment of diabetes, hepatocytes for hepatic failure, adrenal gland cells for Parkinson's disease, cells that produce nerve growth factor (NGF) for Alzheimer's disease, cells that produce factors VIII and IX for hemophilia, and endocrine cells for treating disorders resulting from hormone deficiency, e.g., hypoparathyroidism.

Moreover, by using recombinant DNA methods, so-called "gene therapy," or encapsulating other tissues, it should also be possible to treat patients suffering from chronic pain, cancer (e.g., hairy cell leukemia, melanoma, and renal carcinoma), AIDS (treated by immunological augmentation), Kaposi's Sarcoma (treated by administration of interferon, IL-2, or TNF-α), primary hematologic disorders, patients with long-lasting aplasia, and patients who are myelosuppressed (treated by bone marrow transplantation and aggressive chemotherapy). Uncoated gel beads should also be useful in the treatment of affective disorders, Huntington's Disease, Duchenne's Muscular Dystrophy, epilepsy, infertility, spinal cord injuries, and in wound healing.

Implantation of specific cells can also serve to detoxify or to remove deleterious substances from the circulation. For example, the implantation of appropriate living cells restores normal physiologic function by providing replacement for the diseased cells, tissues, or organs, e.g., in hepatic encephalopathy (produced by liver disease) or uremia (produced by kidney failure).

In each application, a sufficient number of uncoated beads, containing the desired living cells, are implanted into the patient, e.g., surgically or with a syringe. The beads are implanted, e.g., intraperitoneally, for a systemic effect, or into a particular location, e.g., the brain to treat Parkinson's disease, or the spinal cord to treat spinal cord injuries, for a local effect.

The dose of uncoated beads to be used is determined initially from results of in vitro studies. In addition, in vivo results in, e.g., mice, rats, or dogs will facilitate more accurate assessment of required doses, as these tests are generally predictive of efficacy in human patients. For example, spontaneous diabetes in dogs is considered to be similar to type 1 diabetes in man. Soon-Shiong et al., *Transplantation*, 54:769–774 (1992).

The beads are intended to remain in the patient with viable donor cells for extended periods of time up to several months or years. However, if it is determined that the donor cells are no longer viable, e.g., by monitoring the patient's blood for a certain level of the protein secreted by the donor cells, it is a simple task to renew the supply of beads in the patient.

Diabetes Mellitus

To treat diabetes, e.g., in a dog or human patient, the implantable beads preferably encapsulate isolated canine or porcine islets or other cells that produce insulin or insulin-like growth factor 1 (IGF-1). Islets are prepared and encapsulated using procedures described above. Insulin secretory activity of the encapsulated cells or islets is determined both in static culture, e.g., expressed per islet volume, and based on the capability of the islets to respond to graded concentrations of glucose. These values are established as described above. Once the insulin secretion activity of a particular batch of encapsulated islets is determined, the proper number of beads can be determined and implanted into a diabetic

patient. For example, to treat a human patient that requires 20 to 50 units of insulin per day, the total number of beads should be selected to contain a total of about 1.0 to 2.5 million porcine islets. For beads designed to contain, on average, 20,000 islets/ml of gel, the proper dosage would be beads made from 50 to 125 ml of gel.

Hemophilia

Hemophilia is an X-linked hereditary bleeding disorder caused by Factor VIII or Factor IX deficiency. Recombinant methods have now been successfully used to create Factor VIII- and Factor IX-producing cells as described above. Encapsulation in uncoated gel beads and implantation of such cells according to the present invention can thus be used for an improved treatment for hemophilia.

Hepatic Diseases

Hepatocyte transplantation is useful not only for irreversible hepatic failure, but for several disease processes including hereditary enzyme abnormalities, acute hepatic failure, where the ability of the liver to regenerate may still exist, and as a bridge to whole liver transplantation in patients who develop sudden hepatic failure, either because of medical progression or because of rejection-related complications.

Wong and Chang, Biomat. Art. Cells Art. Org., 16:731 (1988), have demonstrated the viability and regeneration of microencapsulated rat hepatocytes implanted into mice. Viable hepatocytes were microencapsulated in alginate-poly-(L-lysine) and implanted intraperitoneally into normal and galactosamine-induced liver failure mice. Eight days after implantation in the mice with induced liver failure, the viability of the encapsulated rat hepatocytes increased from 42% to nearly 100%. After 29 days, the viability of the encapsulated hepatocytes implanted in normal mice also increased from 42% to nearly 100%. By contrast, free rat hepatocytes implanted into mice all died within four or five days after xenotransplantation. The uncoated beads of the invention are well-suited to treat hepatic failure.

Other investigators have shown that microencapsulated hepatocytes continue the synthesis and secretion of many specific proteins and enzymes. Cai et al., *Hepatology*, 10:855 (1989), developed and evaluated a system of microencapsulation of primary rat hepatocytes. Urea formation, prothrombin and cholinesterase activity, the incorporation of tritiated leucine into intracellular proteins, and the immunolocation of synthesized albumin were monitored in culture. Despite gradual decreases in some of these activities, the encapsulated hepatocytes continued to function throughout the 35-day observation period. In addition, Bruni and Chang, *Biomat. Art. Cells Art. Org.*, 17:403 (1989), demonstrated the use of microencapsulated hepatocytes to lower bilirubin levels in hyperbilirubinemia. Microencapsulated hepatocytes were injected into the peritoneal cavity of Grunn rats. Bilirubin dropped from 14mg/100ml to 6 mg/100ml, and remained depressed after 90 days. Again, the uncoated gel beads of the invention can be used as described above to treat these hepatic diseases.

Parkinson's Disease

Parkinson's disease is a neuronal system disease, involving a degeneration of the nigrostriatal dopaminergic system. Experimental work in both rodents and nonhuman primates has shown that transplantation of fetal tissue containing substantia nigra (dopaminergic) neurons from ventral mesencephalon to dopamine-depleted striatum reinstates near-normal dopamine innervation and reduces motor abnormalities. In addition, implantation of adrenal chromaffin cells has been shown to reverse chemically-induced Parkinson's disease in rodents.

Widner et al., *Transplant Proc.*, 23:793 (1991), recently reported evidence of fetal nigral allograft survival and function up to 10 months after transplantation and immunosuppression (cyclosporine, azathioprine, and prednisone) in a human Parkinson's patient. Beginning from the second month after the transplantation, they observed a progressive decrease in limb rigidity, increased movement speed in a number of arm, hand, and foot movements, and prolonged "on" periods (>80% increase) after a single dose of L-dopa.

Thus, transplantation of fetal neural tissue, or cells genetically engineered to produce dopamine and nerve growth factors or other neurotropic factors, should have a great potential as a new therapeutic approach in patients with neurological disorders. However, in the case of transplanted xenogeneic donor tissue, rejection would pose a serious problem, even by the combined approach of using an immunoprivileged site and by employing immunosuppressive drugs. Therefore the uncoated beads of the invention permit a novel approach to this problem, i.e., the delivery of dopamine for the treatment of Parkinson's disease using encapsulated donor tissue harvested from animals or genetically engineered cells.

Alzheimer's Disease

An estimated 2.5 to 3.0 million Americans are afflicted with Alzheimer's disease. The disease is characterized by a progressive loss of cognitive function associated with degeneration of basal forebrain cholinergic neurons. Studies in animals indicate that Nerve Growth Factor (NGF), e.g., brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), available from Regeneron and Amgen, respectively, and other neurotropic factors normally act to support the viability and function of these neuron cells, and that continuous infusion of NGF into the ventricles can prevent injury-induced degeneration of cholinergic neurons as described in Williams et al., *P.N.A.S., USA*, 83:9231 (1986). This treatment correlates with improved cognitive function in rodents with memory impairment as described in Fisher et al., *Neurobiol. Aging*, 10:89 (1989).

These studies suggest that uncoated gel beads containing grafts of recombinant or natural NGF-secreting tissue such as astroglial cells or developing skin, can be used to treat patients suffering from Alzheimer's disease.

Gene Therapy

Gene therapy is an approach to treating a broad range of diseases by delivering therapeutic genes directly into the human body. Diseases that can potentially be cured by gene therapy include diseases associated with the aging population such as cancer, heart disease, Alzheimer's disease, high blood pressure, atherosclerosis and arthritis; viral infectious diseases such as acquired immune deficiency syndrome (AIDS) and herpes; and inherited diseases such as diabetes, hemophilia, cystic fibrosis, and muscular dystrophy.

In one particular example, a favored approach for human gene therapy involves the transplantation of genetically-altered cells into patients, e.g., as described Rosenberg, et al., *New Eng. J. Med.*, 323:570–578 (1988). This approach requires the surgical removal of cells from each patient to isolate target cells from nontarget cells. Genes are introduced into these cells via viral vectors or other means, followed by transplantation of the genetically-altered cells back into the patient. Although this approach is useful for purposes such as enzyme replacement therapy (for example, for transplantation into a patient of cells that secrete a hormone that diseased cells can no longer secrete), transplantation strategies are less likely to be suitable for treating diseases such as cystic fibrosis or cancer, where the diseased cells themselves must be corrected. Other problems commonly encountered with this approach include technical problems, including inefficient transduction of stem cells,

low expression of the transgene, and growth of cells in tissue culture which may select for cells that are predisposed to cancer.

The uncoated particles of the invention are well suited to avoid these problems, because they allow the use of standard human cell lines of, e.g., fibroblast cells, epithelial cells such as HeLa cells, and hepatoma cells such as HepG2, as the implanted cells, rather than requiring the surgical removal of cells from the patient. These cell lines are genetically altered as required by standard techniques and are encapsulated and implanted into the patient. These cell lines are much easier to obtain, culture, and work with than individual patients' cells. Moreover, since the uncoated particles prevent the patient's immune system from recognizing and attacking the implanted cells, any human cell lines can be used, making the technique of gene therapy more universally applicable.

Hypoparathyrodism

Acute and chronic symptoms of hypoparathyroidism result from untreated hypocalcemia, and are shared by both hereditary and acquired hypoparathyroidism. The hereditary form typically occurs as an isolated entity without other endocrine or dermatologic manifestations or, more typically, in association with other abnormalities such as defective development of the thymus or failure of other endocrine organs such as the thyroid or ovary. Acquired hypoparathyroidism is usually the result of inadvertent surgical removal of all the parathyroid glands, and is a problem in patients undergoing operations secondary to parathyroid adenoma or hyperplasia. Hypoparathyroidism has been treated in hypocalcemic rats by the administration of microencapsulated parathyroid cells that served as a bioartificial parathyroid. Parathyroid cells can also be encapsulated in the uncoated gel beads of the invention for use in administration to animal and human patients.

Osteoporosis

The term osteoporosis covers diseases of diverse etiology that cause a reduction in the mass of bone per unit volume. These diseases can be treated by the administration of uncoated gel beads containing cells that secrete insulin-like growth factor (IGF-1), estrogen in postmenopausal woman to reduce the negative calcium balance and decrease urinary hydroxyproline, androgens in the treatment of osteoporotic men with gonadal deficiency, or calcitonin for use in established osteoporosis.

Reproductive Disorders

There are numerous disorders of the ovary and female reproductive tract that can be treated with progestogens, estrogens, and other hormones. These include progestogen, e.g., progesterone, therapy to inhibit pituitary gonadotropins (precocious puberty in girls), and for prophylaxis to prevent hyperplasia in PCOD. Estrogen therapy is used in the treatment of gonadal failure, control of fertility, and in the management of dysfunctional uterine bleeding. Androgens, gonadotropins, and other hormones are used to treat disorders of the testis, e.g., androgen therapy in hypogonadal men, or gonadotropins to establish or restore fertility in patients with gonadotropin deficiency. Accordingly, these diseases can be treated with uncoated beads containing the appropriate hormone-producing cells.

Huntington's Disease

Huntington's disease is characterized by a combination of choreoathetotic movements and progressive dementia usually beginning in midadult life. Distinctive for the disease is atrophy of the caudate nucleus and, to a lesser extent, other structures of the basal ganglia (putamen and globus pallidus). Rodent cells that secrete neurotropic factors have been implanted into the brains of baboons that have a condition similar to Huntington's disease and reversed some of the damaged nerve networks that, in Huntington's patients, lead to progressive loss of control over the body. Similarly, Huntington's disease in human patients can be treated by the administration of uncoated beads that contain human or recombinant cells that secrete the appropriate neurotrophic factors.

Spinal Cord Injuries

The majority of spinal cord injuries result from damage to the surrounding vertebral column, from fracture, dislocation, or both. Treatment of such injuries involves the administration of nerve growth factors such as ciliary neurotropic factor (CNTF), insulin-like growth factor (IGF-1), and neurotropic factors, to enhance the repair of the central and peripheral nervous system. Thus, uncoated gel beads containing cells that secrete such factors, either naturally or through genetic engineering, can be used to treat spinal cord injuries.

Mood (or Affective) Disorders

Mood disorders are a group of mental disorders such as schizophrenia characterized by extreme exaggerations and disturbances of mood and affect associated with physiologic (vegetative), cognitive, and psychomotor dysfunctions. Many mood disorders are associated with medical diseases that can be treated with uncoated gel beads containing the appropriate cells such as hypothyroidism, Parkinson's disease, Alzheimer's disease, and malignancies as discussed herein. In addition, it has been shown that the neurotransmitter 5-hydroxyindol acetic acid (5-HIAA), a serotonin metabolite, is reduced in the cerebral spinal fluid of depressed patients. Deficits in other neurotransmitters such as dopamine and gamma-aminobutyric acid (GABA) have also been identified in patients with major depression. Therefore, uncoated gel beads containing cells that secrete these neurotransmitter are useful to treat these deficiencies.

Motor Neuron Diseases

Degenerative motor neuron diseases include ALS (see above), heritable motor neuron diseases (spinal muscular atrophy (SMA), and those associated with other degenerative disorders such as olivopontocerebellar atrophies and peroneal muscular atrophy. These diseases can be treated by administration of uncoated gel beads containing cells that secrete neurotropic factors like brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3).

Acquired Immunodeficiency Syndrome (AIDS)

AIDS is caused by an underlying defect in cell-mediated immunity due to the human immunodeficiency virus (HIV), and causes persistent constitutional symptoms and/or diseases such as secondary infections, neoplasms, and neurologic disease. Patients can be treated to ameliorate symptoms by immunologic augmentation with uncoated beads that contain cells genetically engineered to secrete, e.g., recombinant human IL-2 (to decrease suppressor cell activity resulting in an increased T cell adjuvant activity); or recombinant human INF-γ (macrophage augmentation). AIDS-related tumors such as Kaposi's sarcoma can be treated with encapsulated cells that secrete human interferon-α, interleukin-2 and tumor necrosis factor (TNF).

μmyotrophic Lateral Sclerosis fLou Gehrig's Disease)

ALS is the most frequently encountered form of progressive motor neuron disease, and is characterized by progressive loss of motor neurons, both in the cerebral cortex and in the anterior horns of the spinal cord, together with their homologs in motor nuclei of the brainstem. ALS can be treated with uncoated beads that contain cells that secrete nerve growth factors such as myotrophin, insulin-like growth factor (IGF-1), ciliary neurotropic factor (CNTF),

brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). Animal studies with these factors (IGF-1 is available from Cephalon, CNTF from Regeneton, and NT-3 from Amgen), have demonstrated that they can stem the degenerative effects caused by nerve damage or disease.

Cancer

In most cases, cancer originates from a single stem cell which proliferates to form a clone of malignant cells. Growth is not properly regulated by the normal biochemical and physical influences in the environment. There is also a lack of normal, coordinated cell differentiation. Cancer cells develop the capacity for discontinuous growth and dissemination to other parts of the body.

Various cancers can be treated according to the invention by the administration of uncoated gel beads containing cells that secrete interferon-α (IFN-α) (for solid tumors, hairy cell leukemia, Kaposi's sarcoma, osteosarcoma, and various lymphomas); recombinant interleukin-2 (IL-2) (for melanoma, renal carcinoma, and Kaposi's sarcoma); tumor necrosis factor (w/IL-2 for Kaposi's sarcoma); recombinant human IFN-α and recombinant human colony stimulating factor-granulocyte macrophage (CSF-gm) (for Kaposi's sarcoma); recombinant human INF-γ (for macrophage augmentation); CSF (for aggressive chemotherapy, bone marrow transplantation, priming of leukemic cells to enhance sensitivity to chemotherapy and to support dose intensification); ciliary neurotropic factor (CNTF) and insulin-like growth factor (IGF-1) (for peripheral neuropathies caused by chemotherapy); adrenal gland cells (for pain relief when injected into the lower spine to secrete natural painkillers) and progestogen-producing cells (for palliation in endometrial and breast carcinoma).

Duchenne's Muscular Dystrophy

Duchenne's dystrophy is an X-linked recessive disorder characterized by progressive weakness of girdle muscles, inability to walk after age 12, kyphoscoliosis (curvature of the spine), and respiratory failure after the fourth decade. This disease can be treated by administration of uncoated beads containing myoblast cells and growth factors. Myoblasts have been injected into young boys with Duchenne's muscular dystrophy to determine whether the cells can supply a structural protein that is missing. Researchers have observed muscle strength improvement in several of the boys.

Epilepsy

The epilepsies are a group of disorders characterized by chronic, recurrent, paroxysmal changes in neurologic function caused by abnormalities in the electrical activity of the brain. In some forms of focal epilepsy, inhibitory interneurons appear to be preferentially lost. Treatment with neurotropic factors and other neuropeptides such as has been found effective. Therefore, the uncoated beads of the invention containing cells secreting these factors can be used to treat epilepsy.

Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, that the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

What is claimed is:

1. A method of implanting a living donor cell into a host animal, wherein said animal is a mammal larger than a rat or mouse, with minimal inflammatory response or rejection of the donor cell by the host animal, said method comprising the steps of

obtaining an uncoated particle consisting essentially of a biocompatible, temperature-independent gel that encapsulates the living donor cell, wherein said uncoated particle provides a molecular weight cutoff that prevents host animal immune cells from entering the particle, and does not prevent entry of host animal IgG and complement into said particle, wherein said particle has a diameter of 700–6,000 microns, and

implanting the uncoated particle into the host animal.

2. A method of claim 1, wherein said donor cell is obtained from an species that is the same as the host animal.

3. A method of claim 2, wherein the host animal is a dog.

4. A method of claim 2, wherein the host animal is a human.

5. A method of claim 4, wherein the donor cell is a genetically altered human cell.

6. A method of claim 1, wherein the donor cell is obtained from an animal species that is different from the host animal.

7. A method of claim 6, wherein the host animal is a dog.

8. A method of claim 6, wherein the host animal is a human.

9. A method of claim 8, wherein the donor cell is a porcine, bovine, or canine cell.

10. A method of claim 8, wherein the donor cell is a pancreatic islet cell.

11. A method of claim 1; wherein the donor cell secretes Factor IX, Factor VIII, an interleukin, an interferon, or an endocrine hormone.

12. A method of claim 1, wherein the donor cell secretes a nerve growth factor, tumor necrosis factor alpha, a neurotropic factor, or a neurotransmitter.

13. A method of claim 1, wherein said gel particle is spherical and has a diameter of from 1,500–3,500 microns.

14. A method of claim 13, wherein said gel particle has a diameter of from 2000 to 4500 microns.

15. A method of claim 1, wherein said gel is an alginate or alginate derivative.

16. A method of claim 15, wherein said alginate is crosslinked with an ion.

17. A method of claim 16, wherein said alginate is crosslinked with a calcium salt.

18. A method of claim 1, wherein said uncoated gel particle is biodegradable.

19. A method of claim 18, wherein a rate of degradation of said gel in said uncoated particle is selected to match a life expectancy of said donor cell.

20. A method of claim 1, wherein said uncoated particle encapsulates an autologous erythrocyte in addition to the donor cell.

21. A method of claim 1, wherein said uncoated particle containing a living cell is treated with a nitric oxide inhibitor prior to implantation.

22. A method of claim 1, further comprising the step of administering a drug to the host animal at a dosage effective to inhibit fibrosis and inflammation around said uncoated particle, but at a dosage lower than that required to achieve immunosuppression when said donor cell is implanted into the host animal without encapsulation.

23. A method of claim 22, wherein said drug is cyclosporin A and is administered at a dosage that achieves a whole blood trough level of less than about 100 ng/ml in the host animal.

24. A method of claim 22, wherein said drug is administered for up to one month after implantation, and is then no longer administered.

25. A method of implanting a living donor cell into a host animal, wherein said animal is a mammal larger than a rat or mouse, with inflammatory response or rejection of the donor cell by the host animal, said method comprising the steps of

suspending the living donor cell in a liquid medium, said medium consisting essentially of water and a biocompatible, temperature-independent liquid gel,

forming a droplet of said liquid medium that contains at least one living cell,

solidifying said droplet to form a gel particle that encapsulates the living cell, whereby no outer coating is formed on said particle, and wherein said uncoated particle provides a molecular weight cutoff that prevents host animal immune cells from entering the particle, and does not prevent entry of host animal IgG and complement into said particle, wherein said particle has a diameter of 700–6,000 microns, and

implanting the uncoated particle into the host.

26. A method of claim 25, wherein said liquid medium contains pancreatic islets.

27. A method of claim 25, wherein said pancreatic islets are contained in said liquid medium at a density of 10,000 to 35,000 islets per milliliter of said medium.

28. A method of claim 25, wherein said liquid medium contains living cells at a density of about 10^5 to 10^8 cells per milliliter of said medium.

29. A method of treating a disease in a patient, wherein said patient is a mammal larger than a rat or mouse, caused by a deficient production of a substance in the patient, said method comprising the steps of

obtaining an uncoated particle consisting essentially of a biocompatible, temperature- independent gel that encapsulates a living donor cell that secretes said substance, wherein said uncoated particle provides a molecular weight cutoff that prevents patient immune cells from entering the particle, and does not prevent entry of patient IgG and complement into said particle, wherein said particle has a diameter of 700–6,000 microns, and

implanting the uncoated particle into the patient in a location and in a manner that allows the living cell to remain physiologically active and secrete said substance into the patient to treat said disease.

30. A method of claim 29, wherein the donor cell is obtained from an animal species that is the same as the patient.

31. A method of claim 30, wherein the patient is a dog.

32. A method of claim 30, wherein the patient is a human.

33. A method of claim 32, wherein the donor cell is a genetically altered human cell.

34. A method of claim 29, wherein the donor cell is obtained from a species that is different from the patient.

35. A method of claim 34, wherein the patient is a dog.

36. A method of claim 34, wherein the patient is a human.

37. A method of claim 29, wherein the donor cell is a porcine, bovine, canine, bacterial, fungal, or plant cell.

38. A method of claim 29, wherein the disease is diabetes and the donor cell is a pancreatic islet cell.

39. A method of claim 29, wherein the donor cell secretes Factor IX, Factor VIII, an interleukin, an interferon, an endocrine hormone, a nerve growth factor, tumor necrosis factor alpha, a neurotropic factor, or a neurotransmitter.

40. A method of claim 29, wherein the disease is diabetes mellitus, hepatic disease, amyotrophic lateral sclerosis, hemophilia, hypothyroidism, Parkinson's disease, acquired immune deficiency syndrome, Duchenne's muscular dystrophy, infertility, epilepsy, Huntington's disease, hypoparathyroidism, a mood disorder, a motor neuron disease, osteoporosis, or Alzheimer's disease.

41. A method of claim 29, wherein the gel particles are implanted into an immunoprivileged site in the patient.

42. An in vivo method of culturing a living cell, said method comprising the steps of

encapsulating the living cell in an uncoated particle consisting essentially of a biocompatible, temperature-independent gel,

inserting said uncoated particle into an animal, and

allowing said animal to thrive, thereby culturing the cell wherein said animal is a mammal larger than a rat or mouse, wherein said particle has a diameter of 700–6,000 microns and wherein said uncoated particle provides a molecular weight cutoff that prevents host animal immune cells from entering the particle, and does not prevent entry of host animal IgG and complement into said particle.

43. An in vitro method of culturing a living cell, said method comprising the steps of

encapsulating the living cell in an uncoated particle consisting essentially of a biocompatible, temperature-independent gel, wherein said particle has a diameter of 700–6,000 microns and wherein said uncoated particle provides a molecular weight cutoff that prevents host animal immune cells from entering the particle, and does not prevent entry of host animal IgG and complement into said particle

placing said uncoated particle into a medium including nutrients and oxygen, and

maintaining a sufficient amount of nutrients and oxygen in said medium to allow the cell to thrive, thereby culturing the cell.

44. A method of manufacturing uncoated, temperature-independent gel particles containing living cells consisting of the steps of

suspending the living cells in a liquid medium, said medium consisting essentially of water and a biocompatible, temperature-independent, liquid gel,

forming a droplet of said liquid medium,

solidifying said droplet to form a gel particle that encapsulates the living cells, whereby no outer coating is formed on said particle, wherein said particle has a diameter of 700–6,000 μm and wherein said uncoated particle provides a molecular weight cutoff that prevents host animal immune cells from entering the particle, and does not prevent entry of host animal IgG and complement into said particle, and

storing said gelled uncoated particles in a nutrient medium to maintain the viability of the living cells.

45. A method of claim 22, wherein said drug is a nonsteroidal anti-inflammatory drug.

46. A method of implanting a living donor cell into a host animal, wherein said animal is a mammal larger than a rat or mouse, with minimal inflammatory response or rejection of the donor cell by the host animal, said method comprising the steps of

obtaining an uncoated particle consisting essentially of a biocompatible, temperature- independent gel that encapsulates the living donor cell, wherein said uncoated particle provides a molecular weight cutoff that prevents host animal immune cells from entering the particle, and does not prevent entry of host animal IgG and complement into said particle,

implanting the uncoated particle into the host animal, and

administering a drug to the host animal at a dosage effective to inhibit fibrosis and inflammation of the uncoated particle, but at a dosage lower than that required to achieve immunosuppression when the donor cell is implanted into the host animal without encapsulation.

47. A method of claim **46**, wherein said donor cell is obtained from an species that is the same as the host animal.

48. A method of claim **46**, wherein the host animal is a human.

49. A method of claim **46**, wherein the donor cell is a genetically altered human cell.

50. A method of claim **46**, wherein the donor cell is obtained from an animal species that is different from the host animal.

51. A method of claim **50**, wherein the host animal is a human.

52. A method of claim **50**, wherein the donor cell is a porcine, bovine, or canine cell.

53. A method of claim **46**, wherein the donor cell is a pancreatic islet cell.

54. A method of claim **46**, wherein the donor cell secretes Factor IX, Factor VIII, an interleukin, an interferon, or an endocrine hormone.

55. A method of claim **46**, wherein the donor cell secretes a nerve growth factor, tumor necrosis factor alpha, a neurotropic factor, or a neurotransmitter.

56. A method of claim **46**, wherein said gel particle is spherical and has a diameter of from 50 to 6000 microns.

57. A method of claim **46**, wherein said gel is an alginate or alginate derivative.

58. A method of claim **46**, wherein said drug is cyclosporin A and is administered at a dosage that achieves a whole blood trough level of less than about 100 ng/ml in the host animal.

59. A method of claim **46**, wherein said drug is administered for up to one month after implantation, and is then no longer administered.

60. A method of claim **46**, wherein said drug is a nonsteroidal anti-inflammatory drug.

61. A method of claim **1**, wherein said particle is other than a sphere.

62. A method of claim **25**, wherein said particle is other than a sphere.

63. A method of claim **29**, wherein said particle is other than a sphere.

64. A method of claim **46**, wherein said particle is other than a sphere.

* * * * *

EXHIBIT 3

US005958404A

United States Patent [19]

Selawry

[11] Patent Number: 5,958,404

[45] Date of Patent: *Sep. 28, 1999

[54] **TREATMENT METHODS FOR DISEASE USING CO-LOCALIZED CELLS AND SERTOLI CELLS OBTAINED FROM A CELL LINE**

[75] Inventor: **Helena P. Selawry**, Rileyville, Va.

[73] Assignee: **Research Corporation Technologies, Inc.**, Tucson, Ariz.

[*] Notice: This patent is subject to a terminal disclaimer.

[21] Appl. No.: **08/660,258**

[22] Filed: **Jun. 7, 1996**

Related U.S. Application Data

[63] Continuation-in-part of application No. 08/485,340, Jun. 7, 1995, Pat. No. 5,849,285, which is a continuation-in-part of application No. 08/421,641, Apr. 13, 1995, Pat. No. 5,725,854, which is a continuation-in-part of application No. 08/211,695, Apr. 13, 1994, abandoned.

[51] **Int. Cl.**[6] **A01N 63/00**; C12N 5/00; C12N 5/02

[52] **U.S. Cl.** **424/93.7**; 435/325; 435/373; 435/404

[58] **Field of Search** 424/93.7; 435/325, 435/373, 404, 284.1

[56] **References Cited**

PUBLICATIONS

Akerstrom et al "Physiolog. Effects of 125 Dihydroxyvit." Am J. Physiol. 262 (6 Part 1). 1992 abstr. only.

Selawry, et al. (Nov. 1991) "Production of a Factor, or Factors, Suppressing IL–2 Production and T Cell Proliferation by Sertoli Cell–Enriched Preparations. A Potential Role for Islet Transplantation in an Immunologically Privileged Site", *Transplantation* 52(5):846–850.

Oonk, et al. (1988) "Insulin–Like Growth Factor I (IGF–I) Receptors on Sertoli Cells from Immature Rats and Age–Dependent Testicular Binding of IGF–I and Insulin", *Molecular and Cellular Endocrinology* 55:33–43.

Whitmore III, et al. (Oct. 1985) "The Role of Germinal Epithelium and Spermatogenesis in the Privileged Survival of Intratesticular Grafts", *The Journal of Urology* 134(4):782–786.

Hofmann, et al. (Aug. 1992), Abstract No. 92347475 "Immortalization of Germ Cells and Somatic Testicular Cells Using the SV40 Large T Antigen", *Experimental Cell Research* 201(2):417–435.

Lejeune, et al. (1993), Abstract No. 93:280614 "Enhancement of Testosterone Secretion by Normal Adult Human Leydig Cells b Co–Culture with Enriched Preparations of Normal Adult Human Sertoli Cells", *Int. J. Androl.* 16(1):27–34.

Berends, et al. (1991), Abstract No. 92:167853 "Significant Improvement of the Survival of Seminoma Cells in vitro by use of a Rat Sertoli Cell Feeder Layer and Serum–Free Medium", *J. Natl. Cancer Inst.* 83(19):1400–1403.

Carreau, et al. (1988), Abstract No. 88:227147 "Stimulation of Adult Rat Leydig Cell Aromatase Activity by a Sertoli Cell Factor", *Endocrinology* 122(3):1103–1109.

Primary Examiner—David M. Naff
Assistant Examiner—Deborah K. Ware
Attorney, Agent, or Firm—Scully, Scott Murphy & Presser

[57] **ABSTRACT**

A method of treating a disease is provided that results from a deficiency of a biological factor which comprises administering to a mammal Sertoli cells and cells that produce the biological factor. A method of treating diabetes mellitus is carried out by transplanting pancreatic islet of Langerhans cells in conjunction with Sertoli cells to create an immunologically privileged site. A method of creating an immunologically privileged site and providing cell stimulatory factors in a mammal for transplants is also carried out. A method of co-localizing islet cells with Sertoli cells and the use of the co-localized product for treating diabetes mellitus is further provided. Further described is a method of creating systemic tolerance to foreign antigens. A method of enhancing the viability, maturation, proliferation of functional capacity of cells in tissue culture is also provided. In addition, a pharmaceutical composition comprising Sertoli cells and cells that produce a biological factor is provided.

50 Claims, 12 Drawing Sheets

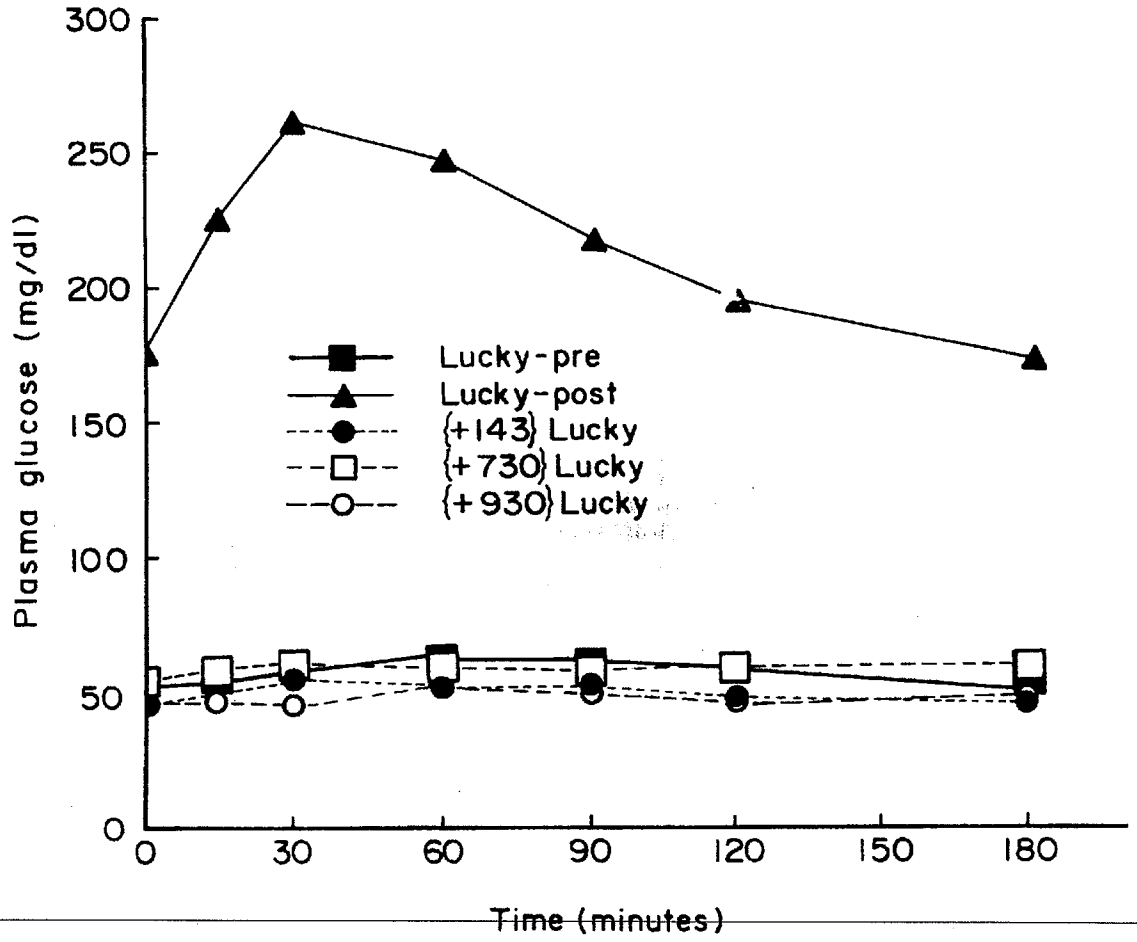

300
250
200
150
100
50
0
Plasma glucose (mg/dl)
0
30
60
90
120
150
180
Time (minutes)
Lucky-pre
Lucky-post
(+143) Lucky
(+730) Lucky
(+930) Lucky

FIG. 1

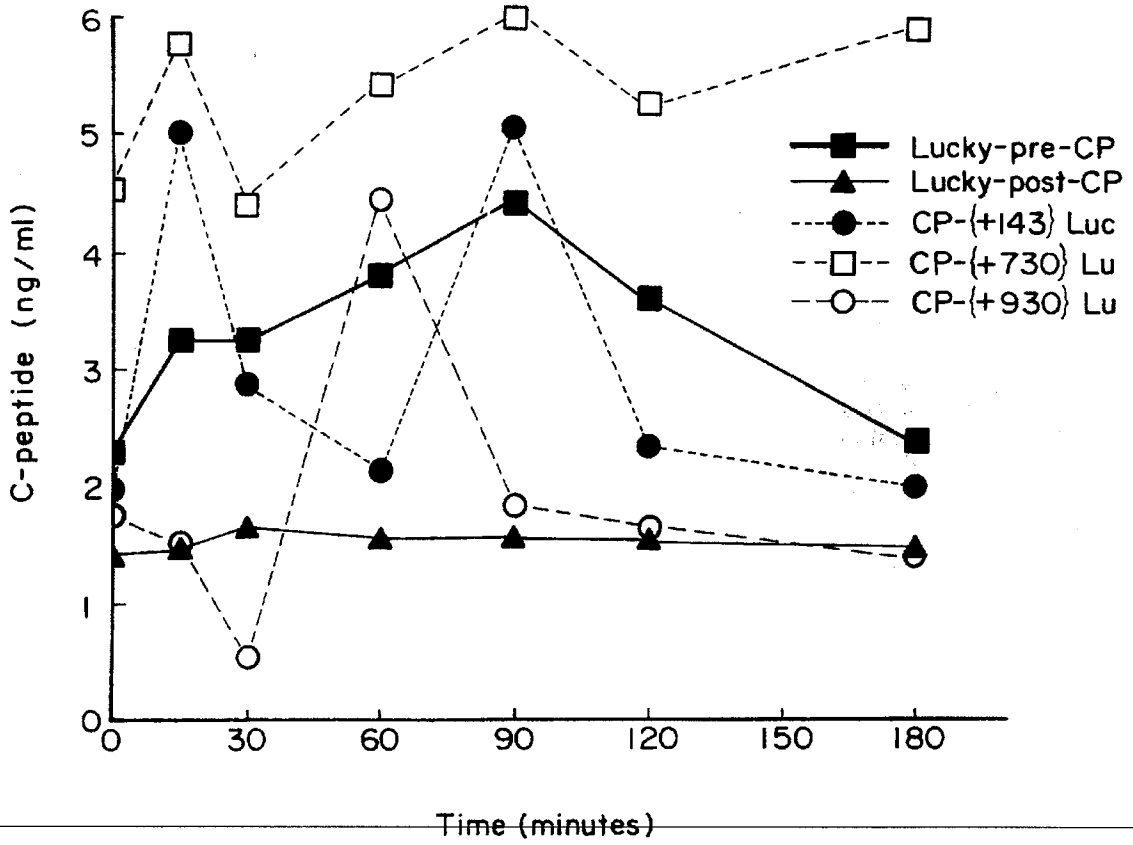

C-peptide (ng/ml)
0
1
2
3
4
5
6
0
30
60
90
120
150
180
Time (minutes)
Lucky-pre-CP
Lucky-post-CP
CP-(+143) Luc
CP-(+730) Lu
CP-(+930) Lu

FIG.2

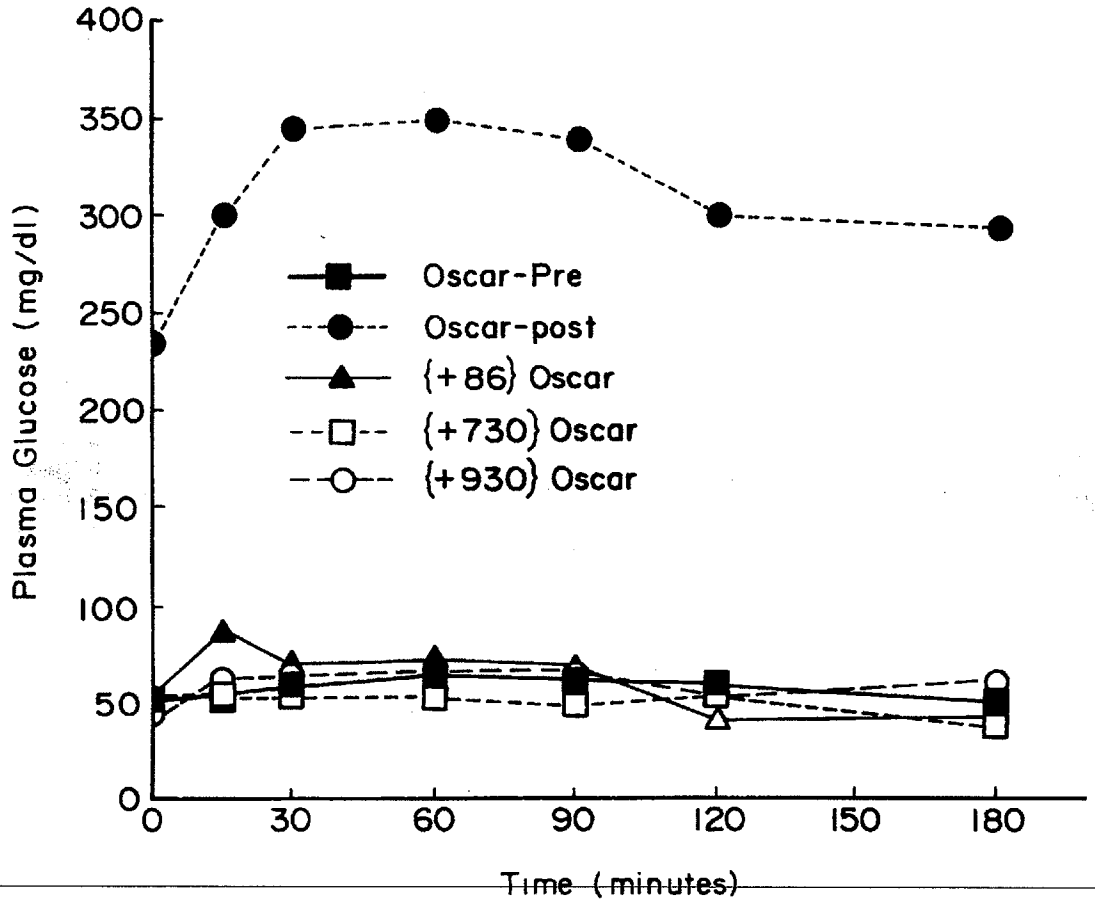

400
350
300
250
200
150
100
50
0
Plasma Glucose (mg/dl)
Oscar-Pre
Oscar-post
(+86) Oscar
(+730) Oscar
(+930) Oscar
0
30
60
90
120
150
180
Time (minutes)

FIG.3

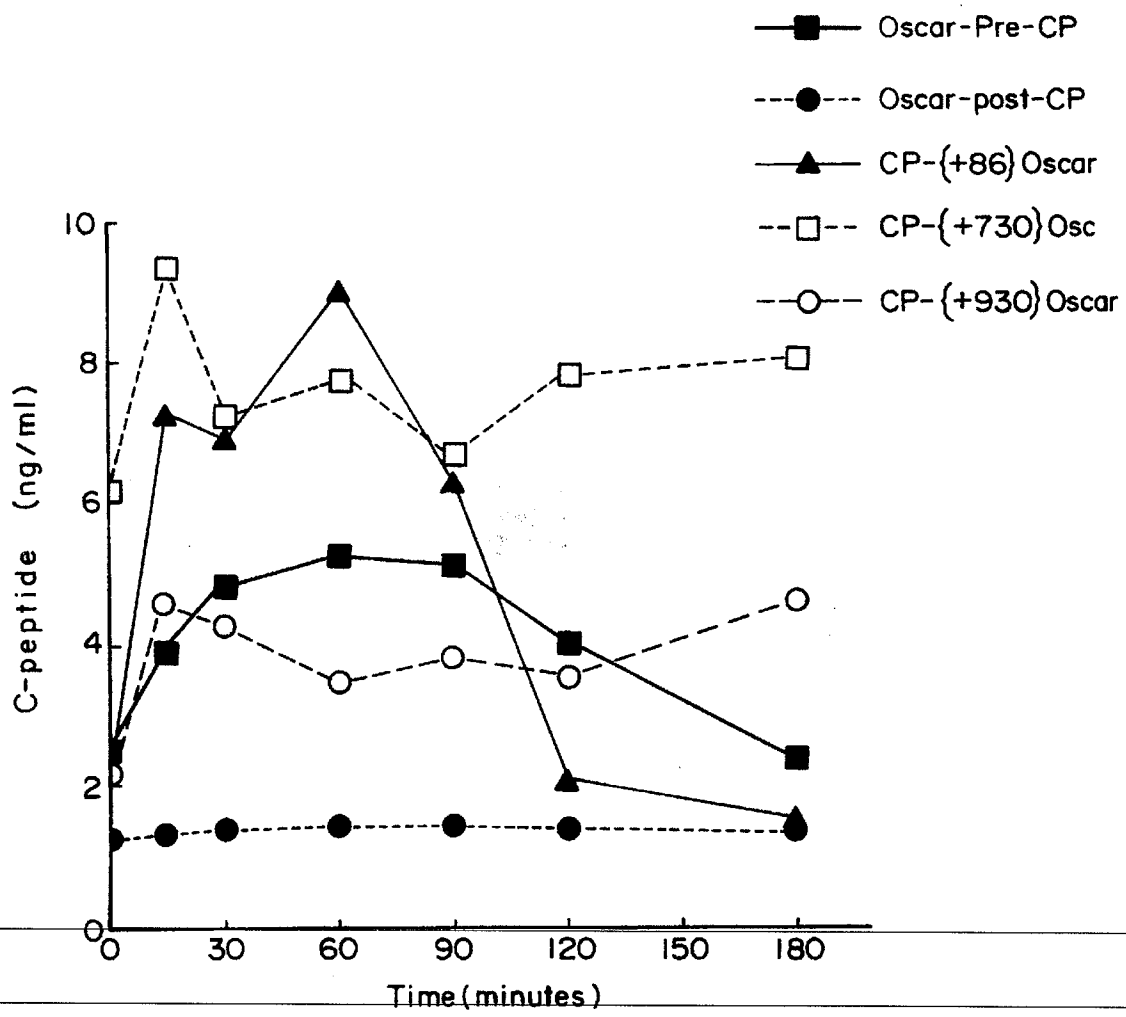

Oscar-Pre-CP
Oscar-post-CP
CP-(+86) Oscar
CP-(+730) Osc
CP-(+930) Oscar
C-peptide (ng/ml)
0
2
4
6
8
10
0
30
60
90
120
150
180
Time (minutes)

FIG. 4

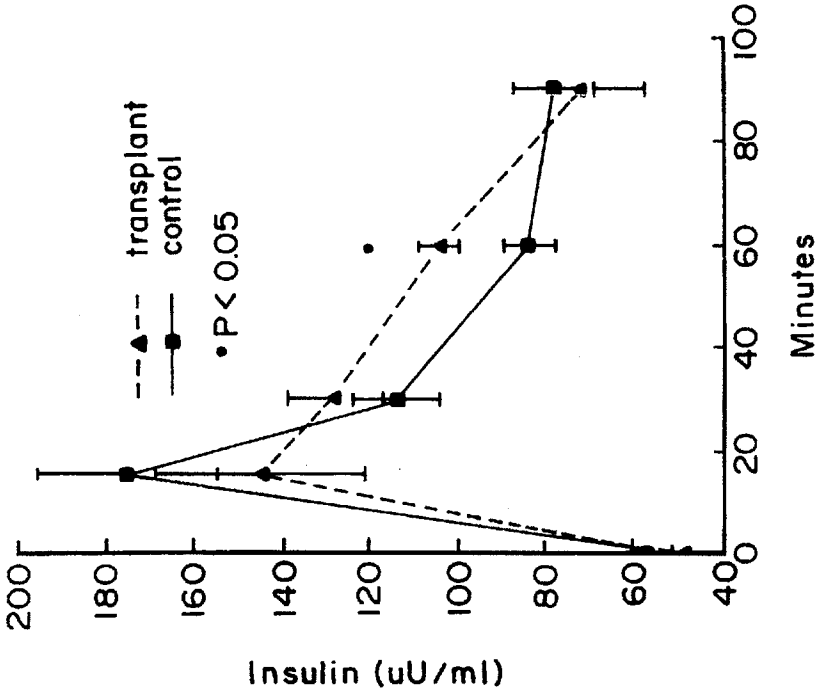

transplant
control
• P < 0.05
Insulin (uU/ml)
200
180
160
140
120
100
80
60
40
0
20
40
60
80
100
Minutes

FIG.5b

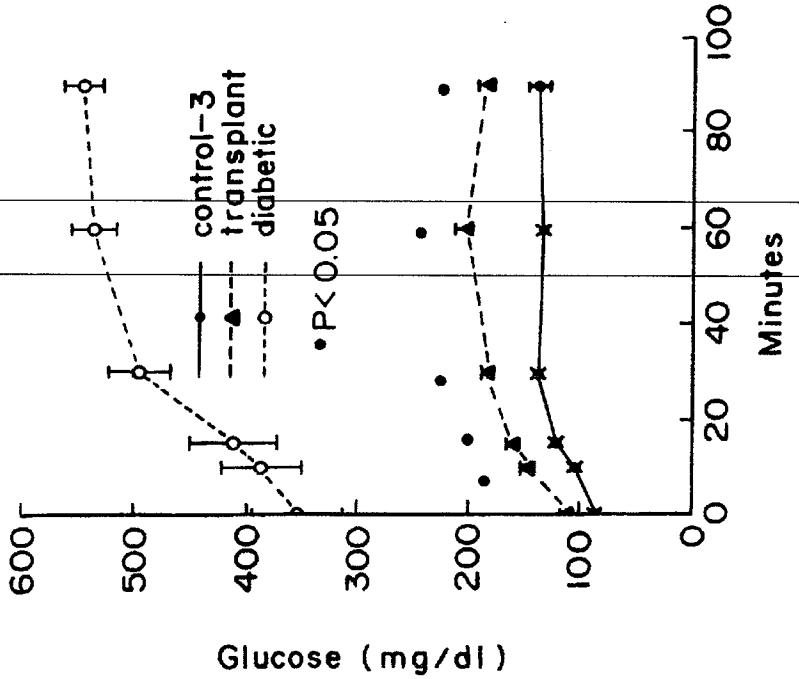

control-3
transplant
diabetic
• P < 0.05
Glucose (mg/dl)
600
500
400
300
200
100
0
20
40
60
80
100
Minutes

FIG.5a

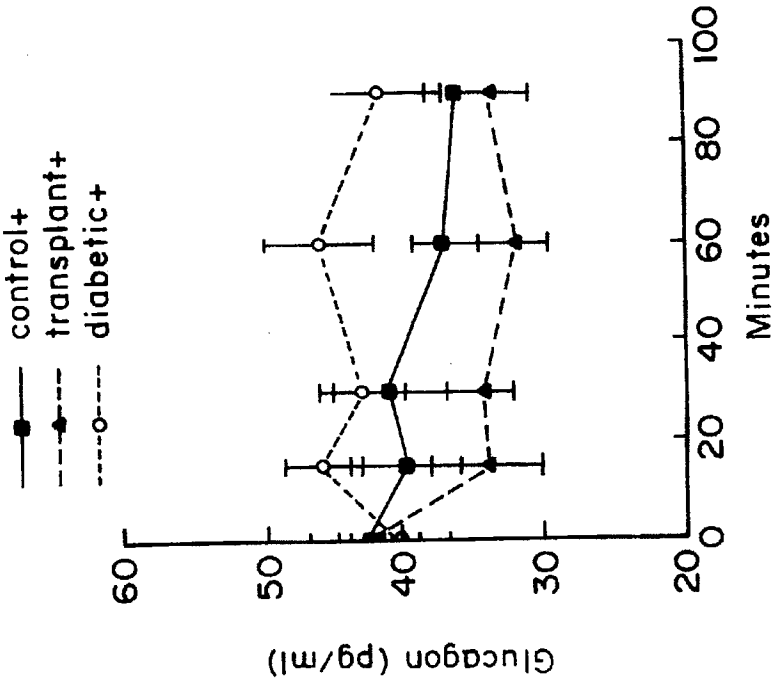

control+
transplant+
diabetic+
Glucagon (pg/ml)
60
50
40
30
20
0
20
40
60
80
100
Minutes

FIG.6b

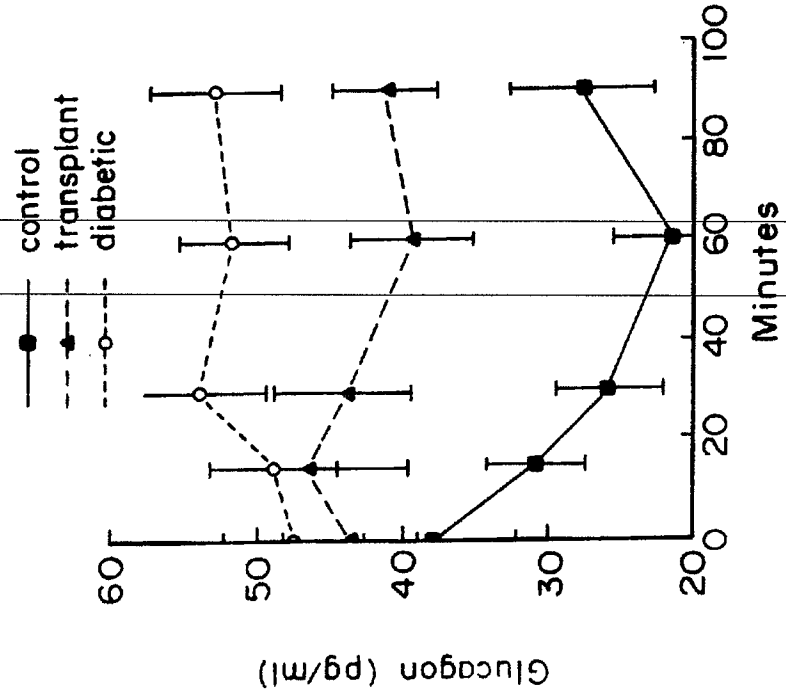

control
transplant
diabetic
Glucagon (pg/ml)
60
50
40
30
20
0
20
40
60
80
100
Minutes

FIG.6a

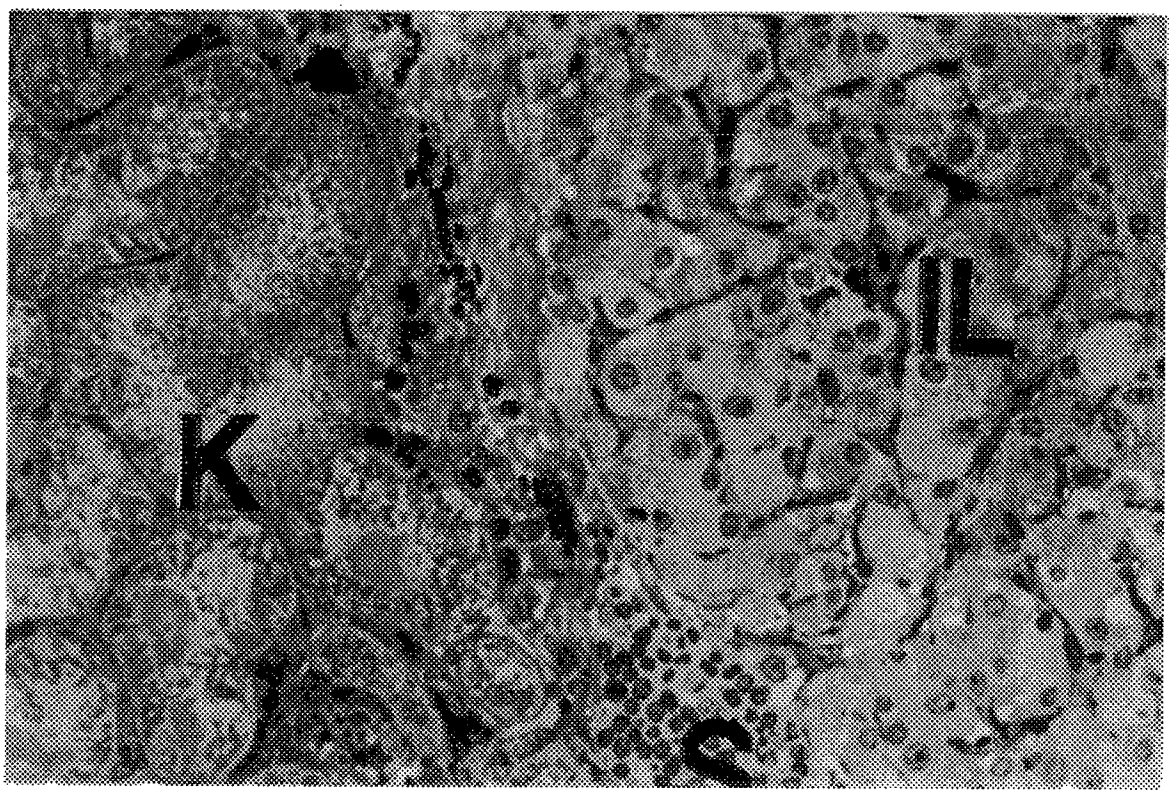


FIG. 7

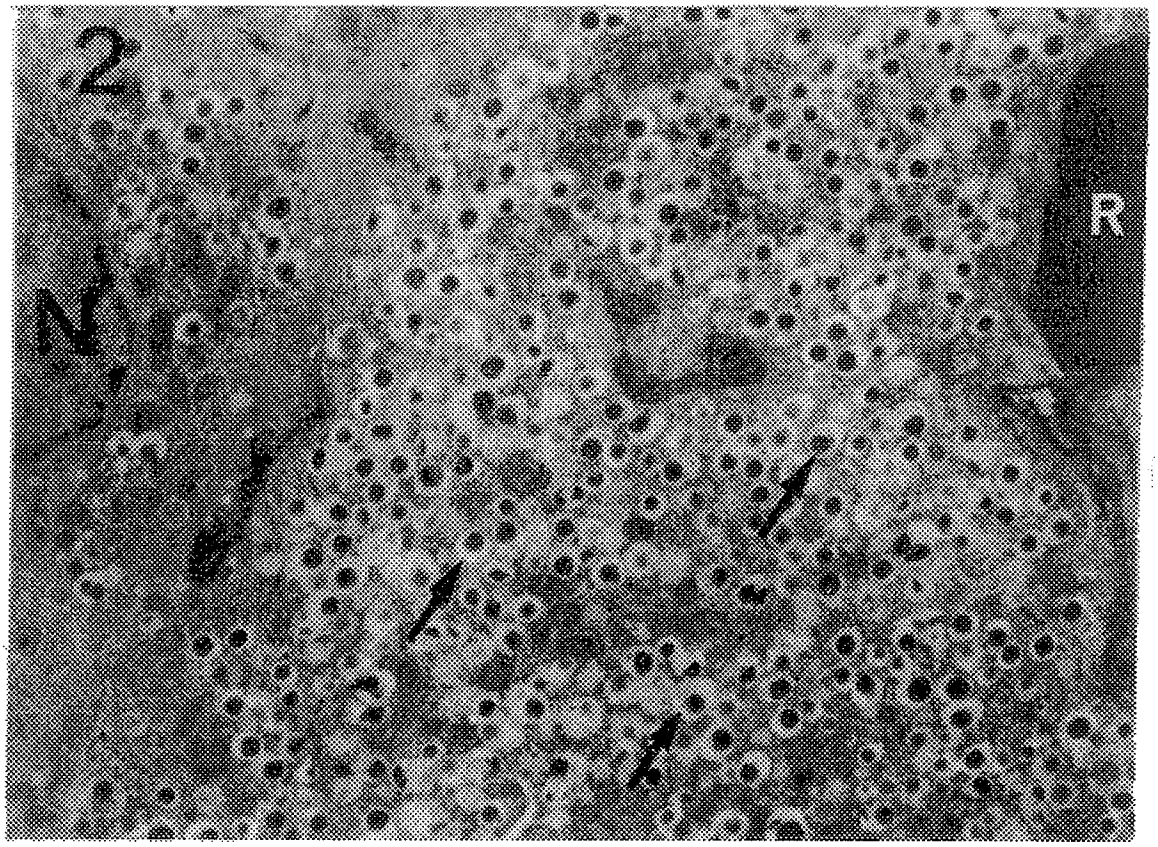


FIG.8

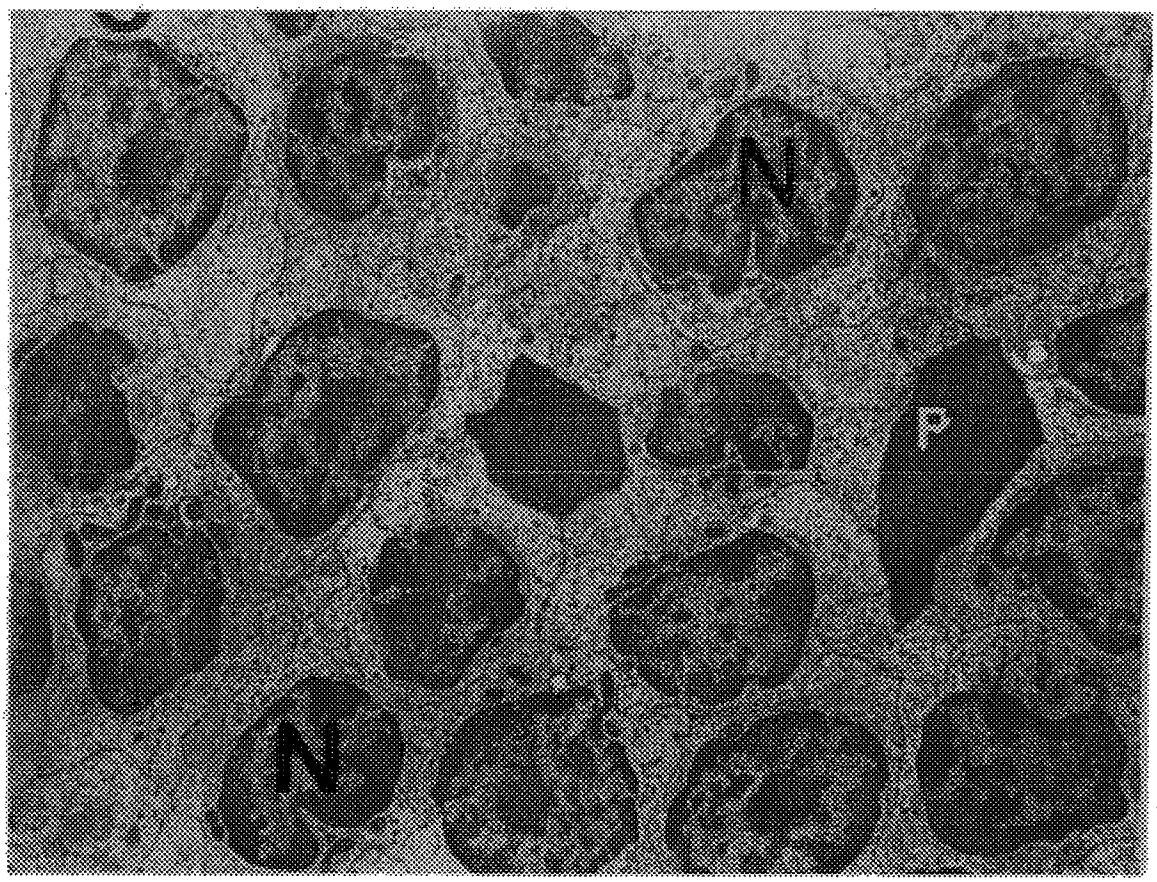


FIG.9

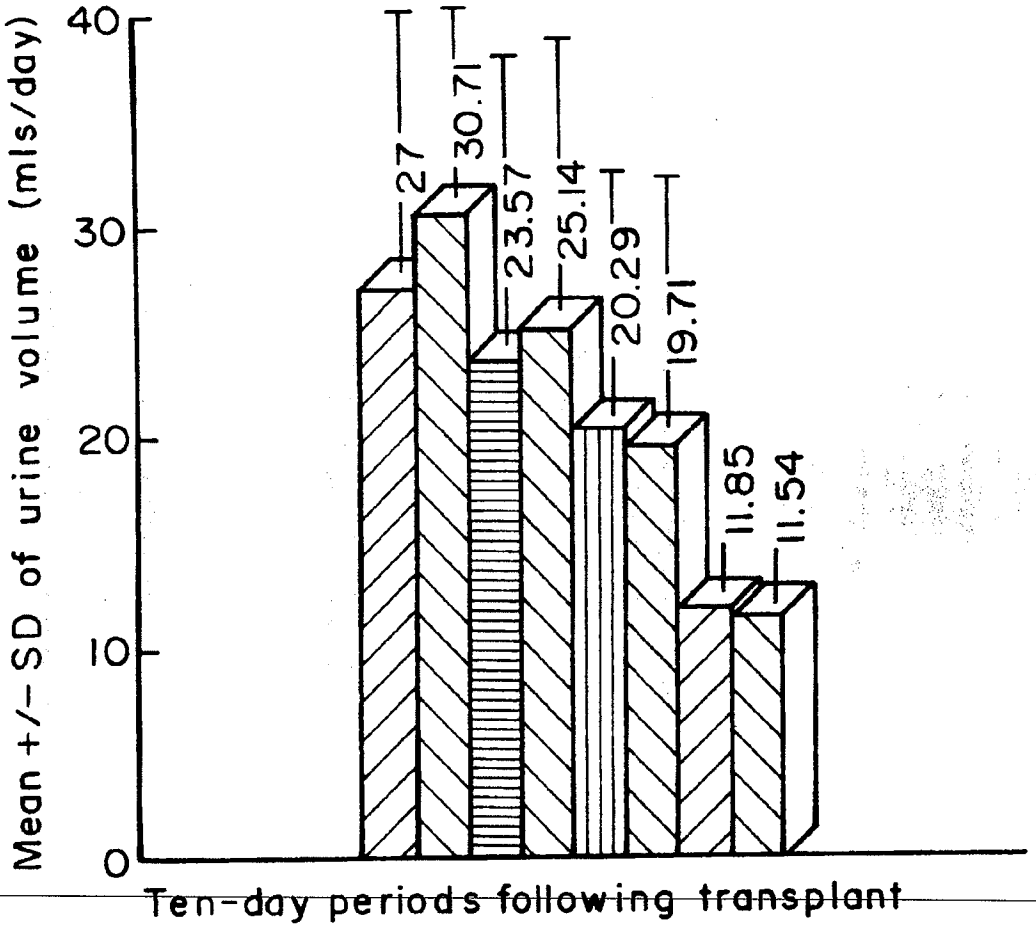

Mean +/- SD of urine volume (mls/day)
40
30
20
10
0
27
30.71
23.57
25.14
20.29
19.71
11.85
11.54
Ten-day periods following transplant

FIG. 10

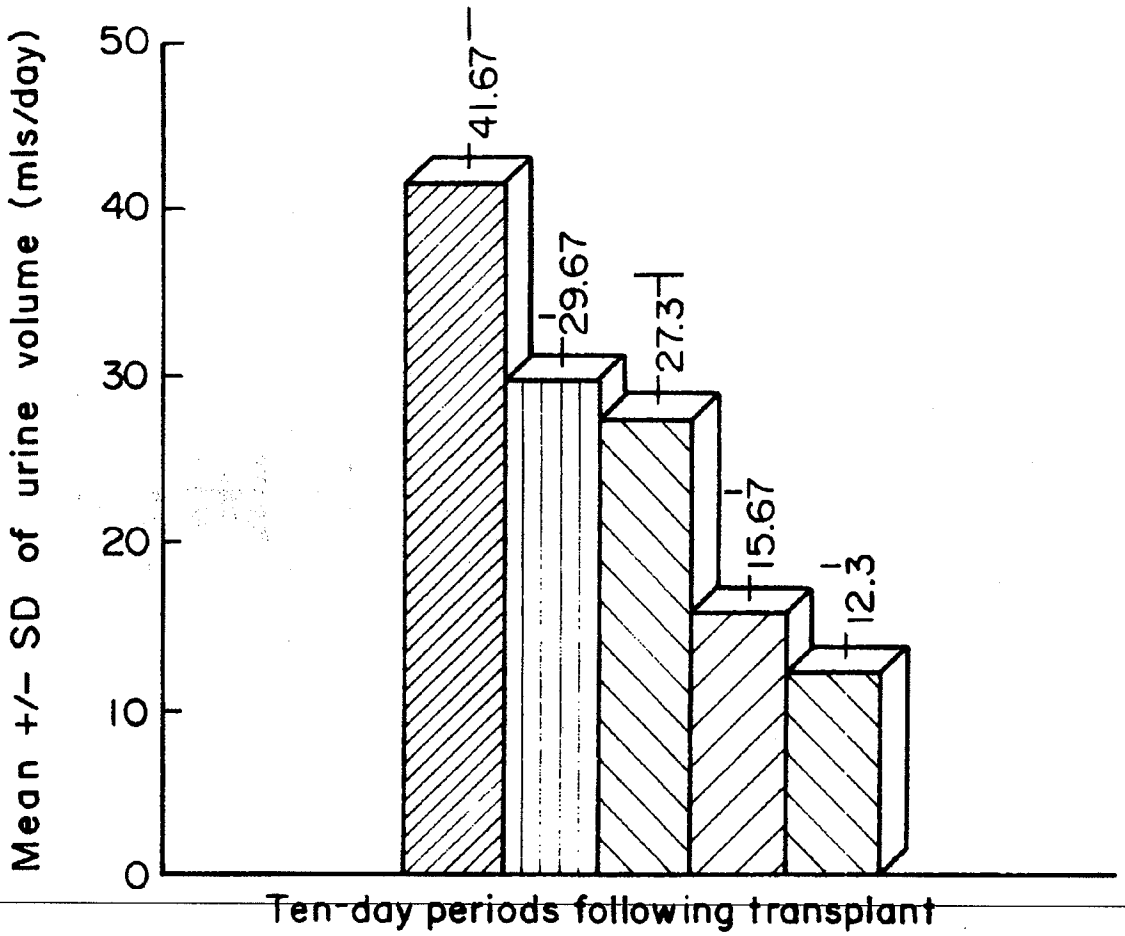

Mean +/- SD of urine volume (mls/day)
50
40
30
20
10
0
41.67
29.67
27.3
15.67
12.3
Ten-day periods following transplant

FIG. 11

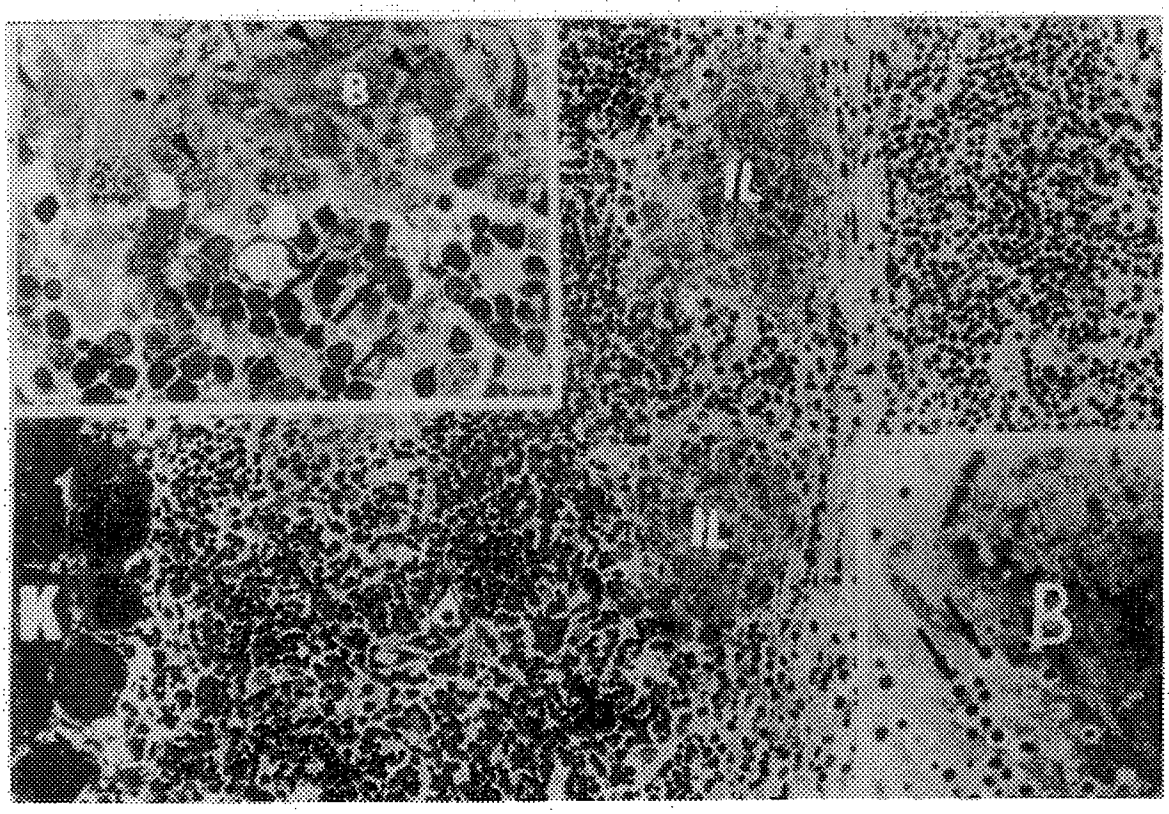


FIG.12

TREATMENT METHODS FOR DISEASE USING CO-LOCALIZED CELLS AND SERTOLI CELLS OBTAINED FROM A CELL LINE

CROSS-REFERENCE OF RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Ser. No. 08/485,340 filed on Jun. 7, 1995, now U.S. Pat. No. 5,849,285, which is a continuation-in-part of U.S. Ser. No. 08/421,641 filed on Apr. 13, 1995, now U.S. Pat. No. 5,725,854 which is a continuation-in-part of U.S. Ser. No. 08/211,695 filed on Apr. 13, 1994 now abandoned.

This invention was made with United States government support under grant DK42421 awarded by the National Institutes of Health. The United States Government may have certain rights in the invention.

FIELD OF THE INVENTION

Transplants of healthy organs or cells into a patient suffering from a disease are often rejected by the body due to an immune response initiated in response to the foreign tissue or cells. The present invention provides a method of cellular transplantation in which an immunologically privileged site is created and cell stimulatory factors are produced, thus alleviating the rejection associated with conventional transplantation therapy. Specifically, the present invention describes a method of treating a disease that results from a deficiency of a biological factor which comprises administering to a mammal Sertoli cells and cells that produce the biological factor. In particular, the present invention describes a method of treating diabetes mellitus by transplanting pancreatic islet of Langerhans cells in conjunction with Sertoli cells to create an immunologically privileged site and to provide pancreatic islet cell stimulatory factors. A method of creating an immunologically privileged site and providing cell stimulatory factors in a mammal for transplants is further described by the present invention. A method of creating systemic tolerance to transplants is further provided by the present invention. The present invention further describes a method of enhancing the maturation, proliferation and functional capacity of cells in tissue culture by coculturing these cells with Sertoli cells. A method of enhancing the recovery rate and viability of frozen cells, and in particular factor producing cells, in tissue culture by co-culturing these cells with Sertoli cells is also described herein. Another aspect of the present invention is directed to a method of co-localizing Sertoli cells with cells that produce a biological factor for treating diseases caused by a deficiency thereof, e.g., encapsulating islet cells which produce insulin with Sertoli cells. The use of the co-localized, e.g., encapsulated Sertoli cells and islet cells for treating diabetes mellitus is further described by the present invention. A pharmaceutical composition comprising Sertoli cells and cells that produce a biological factor is also provided.

BACKGROUND OF THE INVENTION

Certain chronic diseases destroy the functional cells in affected organs. Mammals with such diseases are often unable to produce proteins or hormones necessary to maintain homeostasis and usually require numerous exogenous substances to survive. Transplanting healthy organs or cells into a mammal suffering from such a disease may be necessary to save the mammal's life. This type of therapy is generally regarded as a last alternative to curing an otherwise fatal condition. Such transplants, however, are often rejected by the body due to an immune response initiated in response to the foreign tissue or cells. Presently, the only recourse to combat this immune response is to administer chronic nonspecific immunosuppression agents. Unfortunately, this only trades the complications of one chronic disease with other complications caused by the immunosuppression agent.

One disease which scientists have attempted to treat with organ and/or cellular transplants but have had very limited success is diabetes mellitus. Diabetes mellitus is a prevalent degenerative disease in mammals. It is characterized by a relative or complete lack of insulin secretion by the beta cells within the islets of Langerhans of the pancreas or by defective insulin receptors.

This insulin deficiency prevents normal regulation of blood glucose levels and often leads to hyperglycemia and ketoacidosis. When administered to a mammal, insulin promotes glucose utilization, protein synthesis, formation and storage of neutral lipids and the growth of certain cell types.

In the United States alone there are approximately 13 million diabetics. Of these, 2.6 million are insulin dependent diabetics. *Drug & Market Dev.,* 4:210 (1994). Health care analysts estimate that diabetes costs $92 billion a year resulting from medical costs and lost productivity.

The various forms of diabetes have been organized into a series of categories developed by the National Diabetes Data Group of the National Institutes of Health. Type I diabetes in this classification scheme includes patients dependent upon insulin to prevent ketosis. This group of diabetics was previously called juvenile-onset diabetes, brittle diabetes or ketosis-prone diabetes. Type I diabetes is caused by an autoimmune reaction that causes complete destruction of beta cells.

Type II diabetes is classified as adult-onset diabetics. The diabetic patient may or may not be insulin dependent. Type II diabetes can be caused by a number of factors. For most mammals with Type II diabetes, the beta islet cells are defective in the secretion of insulin.

There are many therapies currently used to treat diabetes, however, each has its limitations. The major problem confronting most patients with diabetes mellitus is that currently available therapies fail to prevent the complications of the disease process. The most common method of treating Type I diabetes in mammals is providing an endogenous source of insulin such as porcine, bovine or human insulin. Insulin injection therapy prevents severe hyperglycemia and ketoacidosis, but does not completely normalize blood glucose levels. This treatment further fails to prevent the complications of the disease process, including premature vascular deterioration. Premature vascular deterioration is the leading cause of morbidity among diabetic patients. Furthermore, complications resulting from long-term diabetes include renal failure, retinal deterioration, angina pectoris, arteriosclerosis, myocardial infarction and peripheral neuropathy.

A second method of treating diabetes is by transplanting the pancreas in conjunction with the administration of chronic nonspecific immunosuppression agents. This treatment is usually given to an individual who has advanced diabetes, such as an individual with kidney failure. Whole pancreas transplantation can be successfully done with a 75% one year survival rate, but surgical transplantation of the pancreas is very difficult. Furthermore, since the entire organ must be donated, the only practicable source is a

deceased donor. In addition, when cyclosporine, the most common immunosuppressive drug used for organ transplants, is administered in a dosage necessary to suppress the immune response, the drug inhibits pancreatic cell function. Furthermore, the steroids that are often administered with an organ transplant often cause the patient to become diabetic.

A third treatment involves transplanting islet of Langerhans cells into the diabetic patient. However, islet transplantation has been generally unsuccessful due to the aggressive immune rejection of islet grafts. (Gray, 1991, *Immunology Letters* 29:153; Jung et al., 1990, *Seminars in Surgical Oncology* 6:122). In particular, successful transplantation of isolated pancreatic islet cells has been very difficult to achieve due to the chronic administration of immunosuppressive drugs required to prevent organ rejection of the cells following transplantation. These dosages of immunosuppressive drugs can cause increased susceptibility to infection, hypertension, renal failure and tumor growth. Furthermore, unlike most organ transplants, islet cells must grow their own blood supply following implantation in the host in order for the cells to survive. Conventional transplantation techniques do not provide the necessary factors to stimulate the production of new blood vessels.

Thus, to successfully transplant cells in a mammal, it is necessary that the cellular transplants are not rejected by the recipient and have the capacity to grow upon transplantation. As a commercial reality, it is further necessary that a sufficient quantity of cells are available for transplantation. Traditionally, the number of cellular transplants have been limited by the inability to adequately collect and store a sufficient number of cells for transplantation. Conventional storage techniques, such as cryopreservation, often damage a large quantity of the stored cells. Porcine islet cells, for example, are extremely fragile and easily dissociate into fragments or single cells upon thawing.

The present invention alleviates many of the problems associated with the current therapies for chronic diseases that destroy the functional cells of vital organs. Specifically, the present invention provides a method of creating systemic tolerance to subsequent transplants in the mammal. Furthermore, the present invention solves the problems associated with the conventional therapies for diabetes mellitus, by providing a method of transplanting pancreatic islets cells into a diabetic mammal, whereby the cellular transplants produce insulin in the diabetic mammal. The present inventor has previously demonstrated extended functional survival of islet cells allografts and xenografts in the testis. (Selawry et al., 1989, *Diabetes* 38:220.) It has been surprisingly discovered in accordance with the present invention that an immunologically privileged site can be created in a mammal by transplanting Sertoli cells to a nontesticular site in a mammal. The newly created immunologically privileged site allows the transplantation and survival of cells that produce biological factors useful in the treatment of diseases, especially diabetes. In addition to creating an immunologically privileged site, the Sertoli cells produce cell stimulatory factors which enhance the maturation, proliferation and functional capacity of cells. Sertoli cells have further been found to enhance the recovery rate and viability of mammalian cells stored by techniques such as cryopreservation.

SUMMARY OF THE INVENTION

The present invention relates to a method of treating a disease that results from a deficiency of a biological factor in a mammal which comprises administering Sertoli cells and cells that produce the biological factor. In a preferred embodiment, the biological factor is a hormone.

In a more preferred embodiment, the disease is diabetes mellitus, the factor producing cells are pancreatic islet cells and the factor is insulin.

In yet another embodiment the cells that produce the biological factors are cells that have been genetically engineered, for example by transformation with a nucleic acid that expresses the biological factor.

The present invention further relates to a method of treating diabetes mellitus in a mammal comprising administering pancreatic islet cells and Sertoli cells. In a preferred embodiment the Sertoli cells and islet cells are administered by transplantation. The Sertoli cells may be isolated from a mammal or they may be derived from a Sertoli cell line, in accordance with the present invention.

Another aspect of this invention is directed to a method of creating an immunologically privileged site and producing cell stimulatory factors in a mammal.

A further aspect of the present invention is directed to a method of creating systemic tolerance to a subsequent transplant in a mammal by transplanting Sertoli cells prior to said subsequent transplant.

Still a further aspect of the present invention provides a method of enhancing the maturation, proliferation and functional capacity of cells in tissue culture by co-culturing these cells with Sertoli cells.

A method of enhancing the recovery rate and viability of frozen mammalian cells and in particular factor producing cells, in tissue culture by co-culturing these cells with Sertoli cells is further provided by the invention described herein.

Another aspect of the present invention is directed to a method of co-localizing, e.g., encapsulating the biological factor producing cells, e.g., islet cells, with Sertoli cells and to the use of the co-localized product for enhancing long-term immunoprotection and nutritional survival of islets and for the treatment of diabetes.

Yet another embodiment of the present invention provides a pharmaceutical composition comprising Sertoli cells and cells that produce a biological factor. In a preferred embodiment the pharmaceutical composition comprises Sertoli cells and pancreatic islet cells and a pharmaceutically acceptable carrier.

The present invention further provides a compartmentalized kit containing Sertoli cells and cells that produce a biological factor. An article of manufacture comprising a packaging material and Sertoli cells contained within the packaging is also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows the glucose responses to oral sustacal tolerance tests done on the monkey "Lucky" at intervals before pancreatectomy (Lucky-pre); after pancreatectomy but prior to transplantations (Lucky-post); and at intervals following transplantation (143 days, 730 days and 930 days, respectively).

FIG. **2** shows the C-peptide responses to an oral sustacal tolerance test at the same time intervals as depicted in FIG. **1**.

FIG. **3** shows the glucose responses to oral sustacal tolerance tests in the monkey "Oscar".

FIG. **4** shows the C-peptide responses in the same animal and at the same intervals depicted for FIG. **3**.

FIGS. 5*a* and 5*b* show the effect of intratesticular islet allografts on serum glucose levels and the insulin responses to oral glucose in spontaneously diabetic BB/Wor dp rats. FIG. 5*a* shows the plasma glucose (mg/dl) concentrations in response to the oral glucose administration of 2 g/kg of a 50% glucose solution in three groups of rats: untreated control Sprague Dawley, transplanted diabetic BB/Wor dp, and insulin treated diabetic BB/Wor dp rats. FIG. 5*b* shows the serum insulin levels in response to the same dose of oral glucose in untreated control Sprague Dawley, and in transplanted BB/Wor dp rats.

FIGS. 6*a* and 6*b* show the effect of intratesticular islet allografts on plasma glucagon secretory responses to oral glucose and a combination of glucose plus glipizide in spontaneously diabetic BB/Wor dp rats. FIG. 6*a* shows the plasma glucagon responses to the oral administration of 2 g/kg of a 50% glucose solution in three groups of rats: untreated control Sprague Dawley, transplanted diabetic BB/Wor dp, and insulin treated diabetic BB/Wor dp rats. FIG. 6*b* shows the plasma glucagon responses to the oral administration of 7 mg/kg of glipizide and 2 g/kg of a 50% glucose solution, administered 30 minutes later, in three groups of rats: untreated control Sprague Dawley, transplanted diabetic BB/Wor dp, and insulin treated diabetic BB/Wor dp rats. Data points are mean±SE of eight animals in each group.

FIG. 7 shows a light micrograph of the pancreatic islets of Langerhans and the isolated rat Sertoli cells transplanted into the renal subcapsular space of a diabetic rat.

FIG. 8 shows an electron micrograph of an individual cell within the transplanted islet.

FIG. 9 shows an electron micrograph of the fine structure of the extra-islet cells labeled "S" in FIG. 7.

FIG. 10 shows the effect of transplantation of piglet islets and Sertoli cells underneath the renal capsule on the mean daily urine output of seven grafted female rat recipients. Each bar represents the mean daily urine output over a ten-day period following transplantation.

FIG. 11 shows the effect of the transplantation of piglet islets and Sertoli cells underneath the skin on the mean daily urine volumes of three rats over a 50-day period.

FIG. 12 shows the light photomicrograph of pig islets of Langerhans and rat Sertoli cells transplanted into the renal subcapsular space of a diabetic rat. IL shows the presence of islands of beta cells (IL) surrounded by an infiltration of small lymphocytes underneath the renal capsule (K); B (upper left) shows at higher magnification that the islands (IL) consist of beta cells and B (lower right) shows that beta cells contain characteristic insulin granules.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to a method of treating a disease that results from a deficiency of a biological factor in mammals which comprises administering to a mammal Sertoli cells and a therapeutically effective amount of cells that produce the biological factor. As defined by the present invention, a biological factor is a protein or nonprotein compound that is necessary for cellular metabolism and homeostasis. In a preferred embodiment, the biological factor is a hormone. Hormone producing cells which can be administered using the method described in the present invention include, for example, pancreatic islet of Langerhans, pituitary, liver, parathyroid, thyroid and ovarian cells.

In accordance with the present invention, the Sertoli cells and the cells that produce the biological factor can be from the same species as the mammal to be treated or from a different species. Further, the Sertoli cells and the cells that produce the biological factor need not be derived from the same species. It has been demonstrated in accordance with the present invention that Sertoli cells from pigs in conjunction with islet of Langerhans from pigs can be used in the treatment of diabetes mellitus in rats. In a preferred embodiment the Sertoli cells are bovine, porcine or human.

Sertoli cells, which are the predominant cells of male testes, used in the method described by the present invention can be separated from other testicular cells such as Leydig cells, peritubular cells and germ cells, using conventional techniques. For example, the testes of a male mammal, such as a boar or ram, are first collected by castration. The testes are then chopped into several pieces and subsequently washed by centrifugation.

Testicular Leydig cells can be removed from the tissue suspension using digestion agents such as trypsin and DNase. The remaining cell suspension is then washed by centrifugation several times. The pellet is resuspended in collagenase, incubated and washed by centrifugation to eliminate peritubular cells within the testes. Testicular germ cells can be removed by incubating the pellet with hyaluronidase and DNase. After several washings by centrifugation, the Sertoli cells are collected to transplant using the method of the present invention.

In accordance with the present invention, the Sertoli cells may be obtained by various methodologies which establish a line of cells derived from primary cultures of mammalian Sertoli cells. In one embodiment the Sertoli cells are immortalized with a chemical or viral transformant, e.g., a temperature-sensitive mutant of the SV40 virus that allows propagation and promotes differentiation of the cells. In another embodiment Sertoli cells are isolated from mammalian tissue by conventional means using various hydrolytic enzymes such as collagenase, hyaluronidase, and the like. The cells are further isolated from the tissue by such conventional methods as filtering and centrifugation to obtain a purified Sertoli cell population. The isolated and purified Sertoli cells are next incubated and conventionally immortalized under conditions known in the art such as treating said cells with a chemical, that transforms the DNA thereof, e.g. a mutagen. Examples include N-nitrosylmethylureas, nitrous acid, hypoxanthine, nitrosamines (see, Freshney, I. R. in *Culture of Animal Cells, A Manual of Basic Technique,* 3 ed., Chapter 15, Wiley-Liss, New York). Alternately, the isolated purified sertoli cells are incubated in a virus-containing medium consisting of, e.g., SV40 virus or polyoma virus and a conventional growth medium such as F12/DMEM, for sufficient time to propagate the Sertoli cells, which are then isolated from the virus. If an infectious virus cell is being utilized, then it is preferred that the virus be attenuated by techniques known in the art. The Sertoli cells may be isolated from the virus or chemical by conventional techniques employing hydrolytic enzymes. To verify that the Sertoli cells are produced by this methodology, the isolated Sertoli cells are optionally screened for the expression of an appropriate isolate for cloning, e.g., on the basis of expression of mRNAs encoding Sertoli cell-secreted proteins.

In accordance with the present invention, a biological factor is a protein or nonprotein compound that is absent, deficient or altered in a disease state. Cells that produce a biological factor can be isolated, for example, by first surgically removing the tissue that produces the factor from a mammal. This tissue is subsequently chopped and digested using conventional techniques. For example, the tissue can

be digested using a collagenase digestion. The particular factor producing cells can subsequently be collected from the digestion mixture using a separation gradient such as a Ficoll gradient. The factor producing cells are then grown in tissue culture in serum using conventional techniques.

In accordance with the present invention, the factor producing cells may be co-cultured with Sertoli cells in tissue culture. Furthermore, factor-producing mammalian cells may be co-cultured, co-localized or co-transplanted with Sertoli cells to enhance the maturation, proliferation and functional capacity of the mammalian cells. It has been demonstrated in accordance with the present invention that the maturation of porcine islet cells was enhanced when these cells were co-cultured with Sertoli cells as evidenced by both the structural integrity and functionality of the porcine islet cells compared to the islet cells cultured without Sertoli cells. Thus, maturation is defined by the present invention as the process by which a cell develops and becomes functional. The enhanced proliferation of porcine islet cells co-cultured with Sertoli cells is evidenced by the larger number of viable, insulin producing cells compared to porcine islet cells cultured without Sertoli cells. Proliferation as used herein, is defined as a process in which cells multiply. The enhanced functional capacity of porcine islet cells cultured with Sertoli cells is evidenced by the greater capacity of the co-cultured islet cells to respond to glucose and glucose plus Forskolin as insulin secretagogues. Functional capacity is defined as the ability of a cell to respond the biological environment and to generate various chemical and biological substances in response to the various substances present in the biological environment (e.g. when islet cells produce insulin in the presence of glucose).

Mammalian cells which can be co-cultured, co-localized or co-transplanted with Sertoli cells as described by the present invention include, for example, germ cells, such as sperm cells, oocytes, ovarian cells and zygotes; endocrine cells, such as pancreatic islet cells, chromaffin, thyroid cells, hepatocytes, parathyroid cells, Leydig cells, follicular cells; hybridoma cells; recombinantly transformed cells; epithelial cells; nerve cells and epidermal cells. In a preferred embodiment, the mammalian cell is a germ cell or endocrine cell. Cells grown in tissue culture can be transplanted into a mammal in conjunction with the Sertoli cells using the methods of the present invention. In accordance with the present invention, factor producing cells may be stored using a variety of conventional techniques, such as cryopreserving the cells prior to growth in tissue culture for subsequent transplantation. It has been observed in accordance with the present invention, that Sertoli cells co-cultured, co-localized or co-transplanted with mammalian cells, and in particular factor producing cells such as islet cells, enhance the recovery rate and viability of the mammalian cells in tissue culture and in particular, enhance the recovery rate and viability of cells that have been previously stored using techniques such as cryopreservation. Moreover, when co-localized with factor producing cells, such as islet cells, Sertoli cells provide immunoprotection and nutritional support when the two cell types are proximally located. Sertoli cells protect the factor producing cells, such as islets, from, inter alia, macrophages, proteins, lymphokines (e.g., IL-1) and toxic factors released by activated lymphocytes. Sertoli cells provide nutritional support for islets through Sertoli secreted growth factors, e.g., IGF (insulin-like growth factor), EGF (epidermal growth factor) and transferrin, thereby permitting the factor-producing cells to survive longer than when the Sertoli cells are not present.

In a preferred embodiment the factor is a hormone, and the hormone producing cells are isolated from a tissue source as described above. For example, insulin-producing cells are isolated from the pancreas. In another preferred embodiment, the factor producing cells are provided by transforming suitable host cells with a nucleic acid capable of expressing the factor of interest. Transformed cells are provided by methods known to one of ordinary skill in the art, and can be found in a myriad of textbooks and laboratory mammals, including Sambrook et al. (1989) Molecular Cloning: A Laboratory Mammal, Cold Spring Harbor Laboratories, Cold Spring, N.Y. If necessary, the nucleic acid encoding the factor of interest can be adapted by methods known to one of ordinary skill in the art to effect secretion of the factor from the transformed cell. The utilization of Sertoli cells in conjunction with the factor producing cells in accordance with the method of the present invention allows the production of an immunologically privileged site and production of cell stimulatory factors in the treated mammal.

The administration of factor producing cells and Sertoli cells into a mammal is accomplished by conventional techniques. In a preferred embodiment, administration is by transplantation and the factor producing cells are injected into the mammal concurrently with or immediately after the injection of the Sertoli cells into the same site. In another embodiment, Sertoli cells and cells producing the biological factor are co-localized and administered to a mammal. As an example, islets and Sertoli cells are co-encapsulated and injected into the mammal intraperitoneally. In another embodiment, co-localized islets and Sertoli cells are transplanted, injected or provided into a biological or non-biological biocompatible material (biomaterial). Examples of a biological biocompatible material include an isolated segment of small intestine with intact circulation, a pouch (e.g. an omental pouch or a gastric pouch etc.), a biocompatible polymeric scaffold, a polymeric sponge or matrix, and the like, prepared pursuant to conventional techniques. On the other hand, a non-biological, biocompatible material includes reticulated thermoplastics such as acylnitrile vinyl chloride copolymer (PAN-PVC), and the like. The biomaterial may be conventionally implanted in a mammal. In accordance with the present invention, an exogenous biological factor may be administered following the transplantation of factor producing cells and Sertoli cells until the transplanted cells produce a therapeutically effective amount of the biological factor. For the treatment of diabetes, for example, insulin may be administered following the transplantation of pancreatic islet cells and Sertoli cells until the transplanted islet cells produce a therapeutically effective amount of insulin.

The Sertoli cells and factor producing cells of the present invention can be transplanted or co-localized using any technique capable of introducing the cells into the mammal such as parenteral administration, subcutaneous administration following surgical exposure to a desired site, biocompatible scaffold, sponge or matrix delivery or intraperitoneal administration. Prior to transplantation, the recipient mammal is anesthetized using local or general anesthesia according to conventional technique. In a preferred embodiment the mammal to be treated is human. In another embodiment the present method of treating disease further comprises administering an immunosuppressive agent such as, for example, cyclosporine, tacrolimus, despergualin and monoclonal antibodies to, e.g., T cells. In a preferred embodiment the immunosuppressive agent is cyclosporine. In another preferred embodiment cyclosporine is administered at a dosage of from 0.5 mg to 200 mg/kg body weight. In a most preferred embodiment cyclosporine is administered at a dosage of from 5 mg to 40 mg/kg body weight.

It has been discovered in accordance with the present invention that administration of Sertoli cells results in the creation of an immunologically privileged site in the treated mammal and in the production of cell stimulatory factors. An immunologically privileged site as defined by the present invention is a site in the mammal where the immune response produced in response to the transplanted cells is suppressed due to immunosuppressive agents produced by Sertoli cells. Immunologically privileged sites are characterized by an available blood supply to provide nourishment for the transplanted cells and a dense tissue to keep the transplanted cells within close proximity of each other. Examples of immunologically privileged sites as defined by the present invention include the renal subcapsular space, subcutaneous facie, the brain and the hepatic portal vein. Cell stimulatory factors are defined by the present invention as factors that enhance the viability of mammalian cells. For example, it has been shown in accordance with the present invention that Sertoli cells increase the rate at which the transplanted factor producing cells vascularize in the transplanted site (i.e. promote angiogenesis). Further, it has been shown by the present invention that these cell stimulatory factors enhance the maturation, proliferation and functional capacity of cells transplanted with Sertoli cells. It is therefore indicated that the Sertoli cells produce cell stimulatory factors which enhance of viability of mammalian cells as evidence, for example, by the increased vascularization rate of the transplanted islet cells. As used herein, viability denotes the number of living cells in a preparation.

In a preferred embodiment, the present invention describes a method of treating diabetes mellitus by transplanting islet of Langerhans in conjunction with Sertoli cells to create an immunologically privileged site. Allografts as used in the present invention describes the transfer of tissues or cells between two genetically dissimilar mammals of the same species. The term xenografts in the present invention describes the transfer of tissues or cells between two mammals of different species.

The transplanted islet of Langerhans cells and Sertoli cells used in the method described by the present invention can be prepared using any number of conventional techniques. For example, islet of Langerhans cells can be prepared from the pancreas of several mammals of the same species. The pancreases are pooled together, chopped up and digested using collagenase. The islet of Langerhans cells can be further isolated using conventional gradients. Once isolated, the islet cells can be grown in culture and then transplanted in conjunction with Sertoli cells to create an immunoprivileged site.

Sertoli cells used in the method described by the present invention can be derived from primary cultures of mammalian Sertoli cells according to the methods known to one skilled in the art including the method of e.g. Roberts et al. (1995) Biology of Reprod. 53:1446–1453, the contents of which is incorporated herein by reference, or the Sertoli cells can be isolated from mammalian male testes. To collect the islet cells, the testes are first chopped into several pieces and then washed by centrifugation. Leydig cells, present in the crude mixture, can be removed from the tissue suspension using digestion agents such as trypsin and DNase. The remaining cell suspension is then washed by centrifugation several times. Following, the pellet may be resuspended in collagenase, incubated and washed by centrifugation to eliminate peritubular cells within the testes. Testicular germ cells can be removed by incubating the pellet with hyaluronidase and DNase. After several washings by centrifugation, the Sertoli cells for transplantation can be collected.

The Sertoli cells can be transplanted to create an immunoprivileged site within a mammal using a variety of techniques. For example, after the mammal is anesthetized, the Sertoli cells can be injected into a tissue mass, thereby creating an immunoprivileged site. The Sertoli cells and factor producing cells of the present invention can be combined using the techniques capable of co-localizing the cells such as microencapsulation inside biocompatible membranes, hydrogels, or reticulated thermoplastics, for example. The co-localized cells can subsequently be injected, transplanted or provided into a tissue mass subcutaneously or into a pouch, e.g. an intestinal pouch, an omental pouch, a gastric pouch, or a biocompatible polymeric scaffold, sponge or matrix consisting of, e.g., polylacetic acid. Once injected, transplanted, or provided, the co-localized product is used to treat diseases caused by a deficiency of the biological factor. For example, the Sertoli cells and islet cells in combination are useful in treating diabetes mellitus.

Sertoli cells are administered in an amount effective to provide an immunologically privileged site. Such an effective amount is defined as that which prevents immune rejection of the subsequently or co-administered cells that produce the biological factor. Immune rejection can be determined for example histologically, or by functional assessment of the factor produced by the cells.

The present invention further provides a method of creating systemic tolerance to a subsequent transplant in a mammal by transplanting Sertoli cells prior to said subsequent transplant as described herein. A transplant as used herein is a mammalian cell, tissue, organelle or organ that is removed from one mammal and placed in the same or different mammal. The subsequent transplant may be made in the same site or a secondary site. A secondary site as used herein, is a transplantation site in the mammal different from the initial transplantation site. Systemic tolerance is demonstrated by various biological phenomena. For example, systemic tolerance results in a diminished destructive immune response to a subsequent allograft or xenograft in a mammal without the administration of prolonged immunosuppressive agents or the co-transplantation of Sertoli cells. In accordance with the present invention, the allograft or xenograft may be any type of transplant, including cells, tissues, organelles or an organ. The types of cells which may be transplanted in accordance with the methods described by the present invention include, for example, endocrine cells, bone marrow cells, hepatocytes or liver cells, nerve cells or brain cells, and islet cells (fetal, neonatal or adult). The types of tissues, organelles or organs which may be transplanted in accordance with the methods described by the present invention include, for example, heart, kidney, pancreas, liver, skin, ligaments, tendons and cartilage.

As demonstrated by the present invention, a mammal may be tolerized (i.e. systemic tolerance may be achieved) by a variety of procedures. For example, systemic tolerance may be achieved by transplanting an allograft or xenograft with Sertoli cells and subsequently transplanting the same type of allograft or xenograft without Sertoli cells or a prolonged administration of immunosuppressive agents. Systemic tolerance may also be achieved by transplanting an allograft of any cell, tissue, organelle or organ without Sertoli cells or prolonged immunosuppressive agents following an initial transplantation of an allograft with Sertoli cells. In a preferred embodiment Sertoli cells are administered in amounts ranging from 10^1 to 10^{10} cells. In a more preferred embodiment, 10^5 to 10^{10} cells are administered.

The cells producing the biological factor are administered in a therapeutically effective amount. The ordinary skilled

artisan can determine the appropriate amount of cells producing the biological factor by methods known in the art. The amount of cells is dependent upon the amount of factor being produced by the cells and the known therapeutically effective amount of the factor necessary to treat the disease. For example, 1 to 1000 islet cells per gram body weight can be administered to treat diabetes using allografts, 20 to 1000 islets per gram body weight are administered using xenografts. In another preferred embodiment, 5 to 100 islet cells per gram body weight are administered to treat diabetes. In a most preferred embodiment, 5 to 20 islet cells per gram body weight are administered, using allografts and 100–1000 islet cells per gram body weight are administered for xenografts.

In another embodiment the present method of treating diabetes further comprises administering an immunosuppressive agent such as, for example, cyclosporine, tacrolimus, despergualin and monoclonal antibodies to, e.g., T cells. In a preferred embodiment the immunosuppressive agent is cyclosporine. In another preferred embodiment cyclosporine is administered at a dosage of from 0.5 mg to 200 mg/kg body weight. In a most preferred embodiment cyclosporine is administered at a dosage of from 5 mg to 40 mg/kg body weight.

More generally, the immunosuppressive agent can be administered for a time sufficient to permit the transplanted islets to be functional. This period extends from the point prior to or immediately following the transplantation of the islets to the point at which the cells are capable of producing therapeutically effective amounts of insulin. In a preferred embodiment, the sufficient period of time to administer an immunosuppressive agent is about 40 to about 100 days following transplantation of the islets. In a more preferred embodiment, the sufficient period of time is about 50–60 days.

A preferred embodiment of this invention is directed to a method of treating Type I and Type II diabetes mellitus by transplanting islet of Langerhans in conjunction with Sertoli cells into the renal subcapsular space.

Unlike the therapies for diabetes described in the prior art, the method of treating diabetes described by the present invention prevents the complications of the disease process and does not result in the adverse side effects associated with conventional diabetes therapy. Furthermore, the method of transplanting islet cells described by the present invention provides the necessary factors for angiogenesis, growth enhancing and increased functional capacity of the islet transplants.

A method of creating an immunologically privileged site in a mammal is further described by the present invention. An immunologically privileged site is created by transplanting isolated Sertoli cells into a mammal in an amount effective to create an immunologically privileged site. In a preferred embodiment, 10^1 to 10^{10} cells are administered. In a more preferred embodiment, 10^5 to 10^{10} cells are administered. In a preferred embodiment the Sertoli cells are transplanted into the renal subcapsular space or subcutaneous facie by injection. In a preferred embodiment the mammal is a human and the Sertoli cells are human or porcine.

A further aspect of the present invention is directed to a method of enhancing the recovery rate and viability of frozen mammalian cells in tissue culture comprising co-culturing the frozen mammalian cell with Sertoli cells. As shown in accordance with the present invention, Sertoli cells produce cell stimulatory factors which enhance the recovery rate and viability of mammalian cells previously frozen. Mammalian cells may be frozen using a widely conventional techniques, including, for example, cryopreservation.

Further contemplated in accordance with the present invention is a method of enhancing the recovery and proliferation of ex vivo cells comprising co-culturing said cells with Sertoli cells for a time and under conditions sufficient to achieve said enhanced recovery and proliferation.

Another aspect of the present invention provides a pharmaceutical composition comprising Sertoli cells and cells producing a biological factor and a pharmaceutically acceptable carrier. In a preferred embodiment the composition comprises Sertoli cells and islet of Langerhans cells and a pharmaceutically acceptable carrier. A further preferred embodiment of the present invention comprises using porcine, bovine or human Sertoli cells and porcine, bovine or human islet of Langerhans cells. As used herein, a pharmaceutically acceptable carrier includes any and all biological and non-biological biocompatible membrane materials. A pharmaceutically acceptable carrier also includes any conventional solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic agents and the like. The use of such media and agents is well-known in the art.

The present invention further contemplates a pharmaceutical composition comprising Sertoli cells and a pharmaceutically acceptable carrier. This pharmaceutical composition, upon administration to a mammal, can be used to treat a variety of diseases, such as for example, autoimmune diseases. Accordingly, the present invention is further directed to treating an autoimmune disease in a mammal comprising administering a therapeutically effective amount of Sertoli cells to the mammal.

The present invention is further directed to a method of enhancing the recovery and proliferation of ex vivo cells comprising culturing said cells with a culture media from a tissue culture containing Sertoli cells for a time and under conditions sufficient to achieve said enhanced recovery and proliferation. As contemplated by the present invention, Sertoli cells are cultured using a conventional tissue culture media as described herein for a time and under conditions sufficient for the Sertoli cells to produce, for example, cell stimulatory factors. The Sertoli cells are then removed from the culture media and the culture media is used in subsequent tissue cultures, for example, as a culture media for sperm cells previously stored by cryopreservation.

Another aspect of the present invention is directed to methods of co-localizing biological factor producing cells, e.g., islets of Langerhans, with Sertoli cells to enhance long-term immunoprotection and nutritional survival of transplanted factor producing cells, e.g., islets. The methods of co-localization include co-localization in an intestinal segment, pouch, e.g. an omental pouch, a gastric pouch or biocompatible polymeric scaffold, sponge or matrix, for example. The method of co-localization in an intestinal segment comprises:

(a) isolating a segment of mammalian small intestine with intact mucosa and intact circulation;

(b) removing the mucosal layer of the small intestine and closing the ends of the isolated segment;

(c) implanting biological factor producing cells and Sertoli cells into the isolated segment; and

(d) fixing the isolated segment to the small intestine.

Methods of co-localizing biological factor producing cells, e.g., islets with Sertoli cells in pouches, such as

omental or gastric pouches are also contemplated by the present invention as described by Amiri, et al. (1990) Arch. Surg. 125:1472–1474 and Bayat, et al. (1995) Surg. Res. Commun. 17:87–91, both of which are incorporated herein by reference. Procedures for co-localizing islets and Sertoli cells in biologically compatible pouches are general and conventionally employed on a case-by-case basis by the skilled artisan in accordance with the present invention.

Procedures for co-localizing islets and Sertoli cells in polymeric scaffolds are readily appreciated by the skilled artisan. It is preferred that the polymeric templates used in accordance with the present invention are biodegradable and comprise a polyvinyl alcohol, e.g., poly-L-lactic acid. Polyvinyl alcohol based templates provide sufficient porosity to permit rapid tissue ingrowth and prevascularization before cell transplantation. In essence, the polymeric template employed in connection with the present invention acts as a matrix or sponge permitting the ingrowth of blood vessels and tissue which facilitates the co-localization of, e.g., islets and Sertoli cells by providing ready access to a nutrient-rich blood supply.

It is preferred that the Sertoli cells are provided in an amount ranging from about 10^1 to about 10^{10} cells. In a more preferred embodiment, Sertoli cells are provided in an amount ranging from about 10^4 to about 10^{10} cells. In yet another preferred embodiment, the factor producing cells are pancreatic islets. The islets are provided in a preferred amount of about 5 to about 200 cells per gram of body weight, and in a more preferred amount of about 5 to about 100 cells per gram of body weight.

A further aspect of the present invention is directed to a method of encapsulating biological factor producing cells, e.g., islets, with Sertoli cells to enhance long-term immunoprotection and nutritional survival of transplanted islets. The method of encapsulation comprises:

(a) suspending a pharmaceutically effective amount of biological factor producing cells, e.g., islets, and Sertoli cells in combination with a gelling effective amount of a first water soluble gelling agent in an aqueous medium which is physiologically compatible with the cells and extruding the islet/Sertoli cell/gelling agent mixture to form a droplet containing the islets and Sertoli cells;

(b) subjecting the product of step (a) to an effective amount of network forming cations to form discrete capsules of sufficient size to encapsulate the islets and Sertoli cells together;

(c) forming a semipermeable membrane around the capsules to obtain a single-walled bead encapsulating the cells; and

(d) contacting the single-walled bead with a gelling effective amount of a second gelling agent so as to form a second semi-permeable membrane encapsulating the product of step (c).

The gelling agent may be any water-soluble material which can be gelled to form a bead. A preferred gelling agent is a water soluble, natural or synthetic polysaccharide gum such as an alkali metal alginate. A preferred gum is sodium alginate. Other gums which may be used include guar gum, gum arabic, carrageenan, pectin, tragacanth gum, xanthan gum or their acidic fractions.

In a preferred embodiment, the Sertoli cells and factor producing cells are encapsulated within an alginate polylysine-alginate semi-permeable hydrogel. It is preferred that the Sertoli cells are derived from bovine, porcine, and human sources and are produced by a cell line in accordance with the present invention. It is preferred that the Sertoli cells are provided in an amount of from 10^1 to 10^{10} cells. In a more preferred embodiment, Sertoli cells are provided in an amount of from about 10^4 to 10^{10} cells. In yet another preferred embodiment the factor producing cells are pancreatic islets. The islets are provided in a preferred amount of about 5 to about 200 cells per gram of body weight, and in a more preferred amount of about 5 to about 100 cells per gram of body weight.

The procedure for the encapsulating of Sertoli cells with cells that produce a biological factor is general, and the procedure will be explained in more detail with respect to Sertoli cells and islet cells, which are exemplary.

In an embodiment of step (a) of the subject method, the first gelling agent is sodium alginate. It is conventionally suspended in an aqueous solution such as a buffer or saline solution containing the islets and Sertoli cells.

By "gelling effective amount" is meant an amount of a water soluble gelling agent capable of binding calcium ions or other ions that interact with the gelling agent to form a network. More specifically, the islets and Sertoli cells in combination are preferably suspended relative to the gelling agent in a ratio of about 1:20 to 20:1 (v/v) and more preferably 1:10 to 10:1 (v/v) and most preferably about 1:10 (v/v). Preferably, the Sertoli cells and islets cells are present in a ratio of about 1:1 (v/v).

The suspension is extruded by techniques commonly used in the art. It is preferred that the suspension is extruded through an air-jet needle. In a preferred embodiment, droplets containing islets and Sertoli cells in association with the alginate are produced by extrusion (1.7 ml/min) through a 22 gauge air-jet needle (air flow 5 l/min).

In an embodiment of step (b) of the subject method, the droplets are subjected to a solution of multivalent cations, such as a solution of calcium salt, e.g., calcium halide, e.g., calcium chloride solution, which form a network within said droplet. The preferred concentration is at least about 0.5% (v/v), and more preferably at least about 1% (v/v), and most preferably ranging from about 1% (v/v) to a saturated solution. In an embodiment, the droplets fall into a beaker containing a saline solution at pH7 of calcium chloride solution, e.g., 10 ml 1.1% $CaCl_2$ in 0.9% saline at pH7. This process continues for a sufficient time until the negatively charged alginate droplets bind calcium and form calcium alginate gel.

In an embodiment of step (c) of the subject method, a membrane is formed around the product of step (b) by subjecting the encapsulated product to polymers, which polymers contain substituents reactive with the gelling agents, especially the acid groups of the gelling agent. The preferred polymers are polyamine acids such as poly-L-lysine (PLL) or polyethylenimine. In a preferred embodiment, the polymer is poly-L-lysine with a molecular weight of about 20 kd. It is preferred that the polymers coat the product of (b) by following the procedure of Goosen, et al. in *Biotech. Bioeng.*, 27: 146–150 (1985), the contents of which is incorporated herein by reference. Without wishing to be bound, it is believed that positively charged poly-L-lysine displaces calcium ions and binds negatively charged alginate, producing a polyelectrolyte membrane.

In an embodiment of step (d) of the subject method the second gelling agent may be sodium alginate which may improve the biocompatability of the capsule in a mammal. The second gelling agent may be added according to the methods employed by Weber, et al. U.S. Pat. No. 5,227,298, incorporated herein by reference. The double walled semi-permeable capsules formulated in connection with the present invention have molecular weight cut-offs in the

range of 50,000 daltons and provide sustained release of factors produced by the encapsulated islets and Sertoli cells. The capsules comprise semi-permeable membranes which function to protect islets from immune responses while simultaneously permitting passage of biological factors produced by islets into the mammal. In still another embodiment of the present invention, co-localized, e.g., co-encapsulated islets and Sertoli cells are connected to a blood supply by techniques known in the art, thereby permitting the free flow of nutrients and inhibiting the influx of molecules produced by the immune system.

The co-localized, e.g., encapsulated cells producing biological factor and Sertoli cells are effective in treating a disease resulting from a deficiency of said biological factor. For example, the co-localized, e.g., encapsulated islet cells with Sertoli cells, are effective in treating diabetes mellitus. Thus a preferred embodiment of this invention is directed to a method of treating diabetes mellitus by co-localizing, e.g., co-encapsulating and transplanting islet of Langerhans into the peritoneal space. This method not only prevents the complications of the disease process, but also reduces the adverse effects associated with other therapies. This method also provides a biological factor in appropriate amounts which are released in a physiological manner.

The present invention is also directed to a kit for treatment of a disease. In one embodiment, the kit is compartmentalized to receive a first container adapted to contain Sertoli cells in an amount effective to create an immunologically privileged site in a mammal, and a second container adapted to contain a therapeutically effective amount of cells that produce a biological factor that is absent or defective in the disease to be treated. In a preferred embodiment, the Sertoli cells are bovine, porcine or human and are provided in an amount of from 10^1 to 10^{10} cells. In a more preferred embodiment, Sertoli cells are provided in an amount of from 10^5 to 10^{10} cells. In another preferred embodiment the cells that produce a biological factor are cells that have been transformed with DNA encoding the factor. In yet another preferred embodiment the cells that produce the factor are pancreatic islet cells. The islet cells are provided in a preferred amount of 5 to 200 cells per gram of body weight, and in a more preferred amount of 5 to 100 cells per gram of body weight.

The present invention further provides an article of manufacture comprising a packaging material and Sertoli cells contained within said packaging material, wherein said Sertoli cells are effective for creating an immunologically privileged site in a mammal, and wherein said packaging material contains a label that indicates that said Sertoli cells can be used for creating an immunologically privileged site in a mammal. The packaging material used to contain the Sertoli cells can comprise glass, plastic, metal or any other suitably inert material.

Unless specified to the contrary, it is to be understood that percentages are by volume.

In order to further illustrate the present invention, the experiments described in the following examples were carried out. It should be understood that the invention is not limited to the specific examples or the details described therein. The results obtained from the experiments described in the examples are shown in the accompanying figures and tables.

EXAMPLE 1

Six male Rhesus monkeys were transplanted with islet allografts in their testes to examine the survival of these transplants. The recipients were made diabetic by means of a near total pancreatectomy, followed two weeks later by an intravenous injection of 35 mg streptozotocin/kg body weight. This procedure resulted in the induction of severe diabetes melitis. Plasma glucose levels were in excess of 400 mg/dl and the animals were ketotic. Malabsorption was prevented by the oral administration of VACUOUS®, one tablet given twice daily before each meal.

Islets were isolated from female Rhesus monkeys. First, the pancreases of five animals were removed, pooled and chopped finely into smaller fragments. After collagenase digestion in a water bath at 37° C., the islets were separated from exocrine tissues and other cellular debris on at least two Ficoll gradients, prepared in tandem. The islets were washed three times by centrifugation in ice-cold Hanks's buffer and then handpicked and transferred in groups of 150 to biologic grade Petri dishes. Each dish contained 6 mL of culture medium CMRL-1066 supplemented with 5% fetal calf serum, glucose at a concentration of 250 mg/dL, penicillin (100 U/mL), and streptomycin (100 μ/mL). Incubation of islets were carried out at 35° C. in 5% CO and air for 4 to 6 days. The islets were transferred to fresh medium at 48 hour intervals.

Viability and counting of the islets were facilitated by means of the uptake of the dye dithizone. Each monkey received an average of about 10^4 islets/kg body weight injected into both testes. In the first three animals the testes were elevated into the abdominal cavity, whereas in the last three recipients the grafted organs were anchored into the inguinal canal. Cyclosporine (CsA) was administered, in varying doses to the first three grafted animals over a 30 day period, whereas the last three hosts were given 7 injections of CsA (20 mg/kg) on days −4 to +3. Oral sustacal tolerance tests were done on day 30, and then at intervals in the normoglycemic animals, as follows.

The monkeys were housed individually in cages and given standard monkey chow and fruit twice daily. In addition, a pancreatic enzyme was mixed with the food since the monkeys had been pancreatectomized to make them diabetic before transplantation.

The night before the test, the animals were fasted for 12 hours. At 8 a.m. the next morning they were then anesthetized and prepared for the test meal. Sustacal was used as the test agent. Sustacal consists of a physiologic mixture of carbohydrates, proteins and fat which closely mimics a standard meal and which is a powerful stimulus for the release of insulin.

Sustacal was injected directly into the stomach of the sleeping animal through a nasogastric tube. Blood samples were then obtained at times 0, 15, 30, 60, 90, 120 and 180 minutes. The samples were centrifuged and the serum stored at −20° C. until measurements for insulin or C-peptide could be carried out. C-peptide is a very sensitive marker for beta cell function. The results are shown in FIGS. **1–4**.

FIG. **1** shows the glucose responses to oral sustacal tolerance tests done on the monkey "Lucky" at intervals before pancreatectomy (Lucky-pre); after pancreatectomy but prior to transplantation (Lucky-post); and at intervals following transplantation (143 days, 730 days and 930 days, respectively).

It can be readily appreciated that the animal became severely diabetic after the removal of his pancreas (Lucky-post). Following transplantation the glucose responses were restored to normal levels at all of the time intervals measured (143, 730 and 930 days following transplantation). Lucky showed no evidence of graft failure. With graft failure glucose levels would become elevated would approach those which were found following his pancreatectomy.

FIG. 2 shows the C-peptide responses to an oral sustacal tolerance test at the same time intervals as depicted in FIG. 1. Following his pancreatectomy the C-peptide responses became blunted indicating a severe diabetes. But following transplantation the levels were not only restored to normal but appeared to show a hyperresponsive pattern of C-peptide release and levels done on day 730 exceed the normal levels at all points measured. The elevated levels might be due to the fact that insulin released from the testis enters the systemic circulation. By contrast, insulin released from the pancreas enters the portal vein and travels immediately to the liver where about 60% is broken down during the first passage. Insulin released into the systemic circulation reaches the liver much later, thus the elevated levels. As was evident with an investigation of the glucose concentrations, the C-peptide responses showed no evidence of failure 30 months following transplantation.

FIG. 3 shows the glucose responses to oral sustacal tolerance tests in the monkey "Oscar". Following the removal of his pancreas he became severely diabetic with elevated glucose levels. Following transplantation of islets the glucose responses became similar to those determined before his pancreas was removed. The glucose levels remain within normal levels 32 months following transplantation.

FIG. 4 shows the C-peptide responses in the same animal and at the same intervals depicted for FIG. 3. The animal became very diabetic following the removal of his pancreas and shows blunted C-peptide responses as a result. Following transplantation and for the next 730 days the C-peptide responses were greater compared with the normals. on day 930 following transplantation the C-peptide responses have become somewhat less compared with the normals. Despite somewhat lower C-peptide levels the animal remains normoglycemic.

This example demonstrates that primates can be successfully transplanted with intratesticular islet allografts without the need for sustained immunosuppression, and that functional integrity of intratesticular islet allografts is maintained for periods exceeding two years with no evidence of graft failure.

EXAMPLE 2

This study examined insulin and glucagon secretory patterns in spontaneously diabetic bb/Wor dp rats transplanted with abdominal, intratesticular, islet grafts. Diabetic, BB/Wor dp, rats received intratesticular islet grafts from MHC-compatible BB/Wor dr rats and no immunosuppression. After a period of 74±15 days, of normoglycdmia, three different groups (controls; BB/Wor dp, transplanted; and BB/Wor dp, insulin treated) were given the following challenges; (1) an oral glucose tolerance test (OGTT), (2) a single oral dose of glipizide, followed by an OGTT, and (3) arginine, by intravenous infusion. The results of this study are shown in Tables 1 and 2 and FIGS. 5 and 6.

TABLE 1

Metabolic Parameters and Immunoreactive Serum Insulin and Glucagon Levels in Control and in Transplanted and Insulin Treated BB/Wor dp Rats

	BB/Wor dp		
	CONTROLS	GRAFTED*	INSULIN TREATED
Plasma Glucose (mg/dl): Prior to Therapy	112 ± 5	502 ± 8+	510 ± 13+
After 2.5 Months	97 ± 4	110 ± 3	350 ± 40#
Duration p.t. OGTT (days)	75 ± 6	70 ± 11	78 ± 19

TABLE 1-continued

Metabolic Parameters and Immunoreactive Serum Insulin and Glucagon Levels in Control and in Transplanted and Insulin Treated BB/Wor dp Rats

	BB/Wor dp		
	CONTROLS	GRAFTED*	INSULIN TREATED
Weight Gain (g)	120 ± 6	105 ± 17	48 ± 14$
Fasting Plasma Insulin (uU/ml)	21.9 ± 3	20.4 ± 2	ND
Fasting Plasma Glucagon (pg/ml)	37.8 ± 5.7	43.4 ± 4.6	47.4 ± 4.9

*Duration of normoglycemia after grafting (days) = 279 ± 25
+ P < 0.0001 vs. control
# P < 0.0001 vs. grafted
$ P < 0.02 vs. grafted

TABLE 2

Pancreatic and Testicular Insulin and Glucagon Content in Control and in Transplanted and Insulin Treated BB/Wor dp Rats

	BB/Wor dp		
	CONTROLS	GRAFTED	INSULIN TREATED
Pancreas (mg)	1573 ± 171	757 ± 122	920 ± 32
Insulin (ug/g)	66 ± 5.03	0.58 ± 0.18	0.76 ± 0.12
Glucagon (ng/mg)	4.1 ± 0.35*	49. ± 0.33**	6.9 ± 0.08
Testes Fractions: (mg)	493 ± 49.6	582 ± 59.2	430 ± 28.0
Insulin (ug/g)	0.0	59.70 ± 0.49	0.0
Glucagon (ng/mg)	0.0	1.4 ± 0.37	0.0

*P < 0.03
**P < 0.08 vs. diabetic, respectively

FIG. 5 shows the effect of intratesticular islet allografts on serum glucose and insulin responses to oral glucose in spontaneously diabetic BB/Wor dp rats. FIG. 6 shows the effect of intratesticular islet allografts on plasma glucagon secretory responses to oral glucose and a combination of glucose plus glipizide in spontaneously diabetic BB/Wor dp rats. This experiment demonstrates that grafted testes in spontaneously diabetic BB/Wor dp rats contain both alpha and beta cells, and that the alpha and beta cells have the capacity to respond to specific secretagogues independently.

EXAMPLE 3

This study investigated the effect of Sertoli cell enriched fraction (SEF) on islet allograft survival in the renal subcapsular space of diabetic rats.

The animals used in this study were PVG rats, weighing between 150–200 g. Diabetes was induced by means of a single intravenous injection of 65 mg/dL of streptozotocin. Only rats with plasma glucose levels in excess of 400 mg/dL were transplanted. Sprague Dawley (S-D) outbred rats were used as islet donors. Either PVG or S-D male rats between 16 and 18 days old were used as Sertoli cell donors.

Islet Preparation

Islets were prepared according to modification of the method of London et al. (1990) *Transplantation,* 49: 1109–1113. The islets were purified on Ficoll gradients, and the isolated cells were then incubated for 4 days at 37° C. in a humidified atmosphere of 5% CO_2, and air prior to use. No special efforts were made to deplete the islets of contaminating passenger leukocytes.

Sertoli Cell-enriched Fraction Preparation

Highly purified preparations of Sertoli cells were isolated form the testes of young males according to the method of Cheng et al. *J. Biol. Chem.,* 26:12768–12779. The testes were removed, chopped into several pieces, and placed in a 50 mL conical tube containing 50 mL of Ham's F12/DMEM media. The pieces were washed once by centrifugation at 800×g for 2 min. The supernatant was aspirated, and the tissue resuspended in 40 mL of media containing 40 mg trypsin and 0.8 mg DNase in a sterile 250 mL Erlenmeyer flask. The flask was placed in 37° C. oscillating incubator at 60–90 osc/min for 30 min. This step removed Leydig cells. The tubules were then transferred to a 50 mL conical tube, and centrifuged at 800×g for 2 min. The supernatant fraction was aspirated, and the pellet resuspended in 40 mL of 1 M glycine, 2 mM EDTA containing 0.01% soy bean trypsin inhibitor and 0.8 mg DNase, and incubated at room temperature for 10 min. This step lysed any residual Leydig cells. The cells were washed by centrifugation for 2 min, and the step repeated twice, or until the media was no longer cloudy. The pellet was resuspended by gentle homogenization with a glass Pasteur pipet in 40 mL of media containing 20 mg collagenase in an Erlenmeyer flask, and incubated at 37° C. for 5 min with 60–90 osc/min. The cell suspension was centrifuged at 800×g for two min, and the pellet resuspended by gentle homogenization with a Pasteur pipet in 40 mL media containing 40 mg collagenase and 0.2 mg DNase, and incubated in an Erlenmeyer flask at 37° C. for 30 min with 60–90 osc/min. The cells were then washed by centrifugation for 2 min, and the process repeated at least three times to eliminate peritubular cells. The, cells were resuspended by gentle homogenization with a Pasteur pipet in 40 mL media containing 40 mg hyaluronidase and 0.2 mg of DNase, and incubated at 37° C. for 30 min with 60–90 osc/min. The cells were pelleted by soft centrifugation for 2 min, and washed at least five times to eliminate germ cells. The resultant SEF was resuspended in 0.25 mL of media, and immediately transplanted into the recipient rat. Each grafted rat received the equivalent of the total amount of Sertoli cells contained in a single testis.

Transplantation of Rats

The diabetic rat was anesthetized with methoxyflurane USP in a sterile hood and the left flank opened to expose the kidney. The Sertoli-enriched fraction containing approximately 5 million Sertoli cells was injected first underneath the renal capsule. The cells could be seen as a milkish bubble underneath the capsule. Immediately afterwards, a total of 10 islets/g of body weight was injected to the same milkish bubble. The needle was retracted slowly to prevent leakage of the grafted cells. Cyclosporine (CsA) was administered subcutaneously in varying doses over a 20-day period to groups two and four. Because the grafted rats responded similarly whether the drug was administered over a 20-day, or over a 3-day period, all of the subsequent groups, including the female rats, were treated with only three injections of 25 mg/kg CsA, given on days 0, +1, and +2, relative to the graft. The rats received no other therapy.

A total of 36 male and 21 female PVG rats were divided into six different treatment groups: Group 1, the control group, consisted of 6 male rats grafted with only islets from S-D donor rats. They received neither SEF nor CsA. Group 2 consisted of 10 rats grafted with a combination of islets from S-D rats and CsA postransplantation, but no SEF. Group 3 consisted of a total of 10 rats grafted with a combination of islets from S-D and SEF from PVG donor rats, but no CsA postransplantation. Group 4 consisted of 10 rats grafted with a combination of islets from S-D donors, SEF from PVG donors, and CsA postransplantation. Group 5 consisted of 11 female rats grafted with the same combination of cells as depicted for Group four. Group 6 consisted of 10 female rats grafted with a combination of islets and SEF, both cell types from S-D donors, and CsA postransplantation.

Posttransplantation Evaluation of Rats

The grafted rats were transferred to metabolic cages, and plasma glucose levels were obtained at weekly intervals. Urine volumes and urine glucose contents were obtained at daily intervals. A rat was considered cured of the diabetic process if the following criteria were met: A random plasma glucose level ≦150 mg/DL; glycosuria; and immediate reversal to hyperglycemia following surgical removal of the grafted kidney.

To determine if any of the rats had become unresponsive to their grafts, normoglycemic rats were challenged with a secondary islet allograft consisting of at least 500, freshly prepared, Sprague Dawley islets which were injected into the contralateral renal subcapsular space. No immunosuppression was given following the challenge.

To examine the impact of the transplantation of SEF on fertility of the female rats, normoglycemic animals of longer than 30 days were mated with PVG males. Metabolic parameters, as outlined above, were closely monitored, as was the course of their pregnancies.

Structural Analysis of Grafted Tissue

A total of five successfully grafted rats were nephrectomized at intervals following transplantation. Wedge sections of renal tissue, obtained from sites at which islets and SEF had been injected, were prepared for examination by light and electron microscopy, as previously described by Cameron et al. (1990) *Transplantation,* 50:649–653. Briefly, the tissue wedges were immersion-fixed with 5% glutaraldehyde in 0.1 M collidine buffer for 1 h, washed in buffer, and postfixed for 1 h with 1% osmium tetroxide in 0.1 M buffer. Small tissue blocks were cut from the wedges, and dehydrated through a graded series of ethyl alcohols, transferred to propylene oxide, and embedded in Epon 812/Araldite plastic resin. Thick (0.5 μm) and thin (900 mg) sections were strained routinely with toluidine blue and uranil acetate/lead citrate, respectively, for structural analysis by light and electron microscopy. The results are shown in Table 3 and FIGS. **7–9**.

TABLE 3

Effect of Sertoli Cells on Islet Allograft Survival in the Non-Immunologically Privileged Renal, Subcapsular Site

Group (n)	Gender	Sertoli Cell (donor origin)	CsA	Duration of Normoglycemia (days) Individual Responses
1 (6)	Male	–	—	0, 0, 0, 0, 0, 0
2 (10)	Male	—	+	0, 0, 0, 0, 0, 0, 0, 130 > 441, >445
3 (10)	Male	+(PVG)	–	0, 0, 0, 0, 9, 10, 12, 13, 13, 14
4 (10)	Male	+(PVG)	+	19, 76, 58*, 84*, 167*, 127†, 139†, >418†, >422 , >425†
5 (11)	Female	+(PVG)	+	7, 11, 14, 28, >287†, >305†, >306†, >308†, >441†, >447†, >457†
6 (10)	Female	+(S-D)	+	8, 10, 96*, 128*, >168, >172, >184, >193, >193, >196

*Nephrectomized

†Challenged with a Secondary Islet Allograft

Group 1: None of the six rats grafted with islets alone, without either SEF or CsA, became normoglycemic.

Group 2: Three of 10 rats grafted with islets and treated with CsA became normoglycemic for more than 100 days. The 3 normoglycemic rats were challenged with a secondary graft on days 116, 192 and 197, respectively. One rat reverted to hyperglycemia on day 130, while 2 remained normoglycemic.

Group 3: Initially 6 of the 10 rats grafted with islets and SEF, but no CsA, became normoglycemic, but all of them reverted to hyperglycemia by day 14.

Group 4: All 10 of rats grafted with a combination of SEF and islets, and also given CsA became normoglycemic. Two reverted spontaneously to diabetes on days 19 and 76, respectively. Three were nephrectomized on days 58, 84 and 167 following transplantation. All 3 of these rats became hyperglycemic within the next 24 h. The remaining 5 rats were challenged with a secondary islet allograft on days 119, 129, 280 342 and 400, respectively. Of these, the first 2 reverted to diabetes on day 127 and 139, respectively, while the latter 3 remained normoglycemic.

Group 5: All 11 of the female rats grafted with a combination of islets and SEF, and then given CsA, became normoglycemic. of these, 4 reverted spontaneously to hyperglycemia by day 28. Of the 7 normoglycemic rats who were mated with male PVG rats, 6 became pregnant, and of these, 8 had litters varying between 1 and 10 pups. They were able to nurse the pups successfully. A total of 7 of the long-term surviving females were challenged with secondary islet allografts at least 200 days following transplantation. None of them reverted to hyperglycemia.

Group 6: of the 10 rats grafted with islets and SEF from the same donor strain of rat, all 10 became normoglycemic. Two reverted to hyperglycemia by day 10. A nephrectomy to remove the graft was done on 2 of the long-term surviving rats on days 96 and 201, respectively. Both reverted to hyperglycemic immediately within the next 24 h.

Tissue Morphology

Renal tissue obtained from the long-term grafted kidney appeared structurally normal by light microscopy (FIG. 7). Transplanted islets in this organ were immediately subjacent to the kidney capsule, and also appeared structurally normal. They displayed tissue and cellular architecture identical to islets in situ (FIG. 7). Individual islet cells were partitioned into cell clusters by thin connective septa containing small vessels and capillaries (FIG. 7). It appeared that most of the islet cells contained secretion granules. When resolved by electron microscopy, islet cells were identified as the β-cell type by the inclusion of ultrastructurally distinctive, and unique insulin-containing secretion granules (FIG. 8). All β-cell clusters observed were in close proximity to intra-islet capillaries (FIG. 8).

There was a high density of cells between, and directly adjacent to, the transplanted islets and renal parenchyma. By light microscopy, they did not appear to be islet cells, kidney cells nor cells or blood origin (FIG. 7). When observed by electron microscopy, these cells were similar in ultrastructure to Sertoli cells in that their nucleic were irregular in profile, and contained deep nuclear clefts, distinctive nucleoli were often present, and mitochondrial structure was dense. Although these cells did not retain the typical polarity of Sertoli cells in vivo, they were, however, identical in appearance to Sertoli cells in vitro, when the cells are not plated on a basement membrane substrate. The cells were not associated with a basement membrane, and appeared randomly organized (FIG. 9). Cells showing ultrastructural features of either germ or Leydig cells were not observed.

This example demonstrates that an immunologically privileged site for transplantation of isolated islet can be created in male and female diabetic recipients by transplantation of Sertoli cells without the need for sustained immunosuppression.

EXAMPLE 4

This study determined the survival of discordant islet xenografts in various nonimmunologically privileged organ sites in experimental animals.

Islets were prepared from young piglets as follows: Male piglets not weighing more than 2.2 kg were used exclusively. The piglet was anesthetized and following exsanguination both pancreas and testes were harvested under sterile conditions. A collagenase solution consisting of 2 mg/ml of collagenase type XI (Sigma) was injected directly into the pancreas. The pancreas was incubated at 37° C. for 17 minutes and the digested tissues washed three times by means of centrifugation and aliquots of 1 ml each transferred to Petri dishes. The islets were incubated at 32° C. in tissue culture media 199 supplemented with 10% horse serum for six days.

On day seven the cultured islets were collected in batches of ±4,000 and cryopreserved using a standard protocol. The cells were stored in liquid nitrogen at 96° C. for periods varying between two and four weeks. The islets were removed from the liquid nitrogen and thawed using an established procedure. The thawed islets were transferred to Petri dishes and co-cultured with pig Sertoli cells for three days at 32° C. in the same 199 culture media as described above. Earlier studies have shown an improved survival rate of thawed islets cultured in the presence of Sertoli cells.

On day three following thawing the islets were hand-picked and counted and a total amount of 12 islets/g of body weight transplanted into female diabetic Sprague Dawley rats. A total of 5 million Sertoli cells procured from the piglet testes were grafted simultaneously into the same location. The organ sites to be tested for the grafting of islets include: a] the renal subcapsular space, b] subcutaneously, and c] the liver. Following transplantation, the rats were treated with cyclosporine as follows: 25 mg/kg for 7 days: 15 mg/kg for 5 days; 10 mg/kg for 5 days; 5 mg/kg for an additional 13 days. On day 30 the drug was discontinued.

To demonstrate viability and functional integrity of isolated piglet islets the following studies were done: a) staining of Cells with dithizone, a stain is highly specific for insulin; b) staining of cells with 0.4% trypan blue which indicates viability of the islets; and c) culturing of batches of 5 islets in the presence of insulin secretagogues such as low and high glucose concentrations at specified intervals following culturing, cryopreservation and thawing. The results are shown in Table 4.

TABLE 4

Insulin Secretion (micro-units/ml) from Incubated and from Cryopreserved-Thawed Islets Done on Days 3, 7 and 14 of Culturing, Respectively

	3 DAYS	7 DAYS	14 DAYS
INCUBATED ISLETS PRIOR TO CRYOPRESERVATION:			
a) Low Glucose (90 mg/dl)	15.3 ± 3.8	21.8 ± 1.1	17.29 ± 2.4
b) High Glucose (300 mg/dl)	32.2 ± 5.4	37.14 ± 3.4	23.3 ± 1.8
CRYOPRESERVED AND THAWED ISLETS:			
a) Low Glucose (90 mg/dl)	14.52 ± 2.8	7.13 ± 1.3	5.38 ± 2.02
b) Low Glucose + Sertoli Cells	10.31 ± 2.8	9.17 ± 2.6	8.38 ± .41

TABLE 5

Yield of Porcine Islets
Following 1, 3 and 7 Days of Culture and the
Percentage of Islets Lost During 7 Days of Culture

Pig. No.	BW (kg)	Panc. W g	D1 Islets/ g panc.	D3 Islets/ g panc.	D7 Islets/ g panc.	Islet Loss % D7/D1
1	1.6	1.79	36,536	31,659	27,212	26%
2	2.0	1.89	37,272	32,962	27,883	25%
3	2.3	2.46	29,268	26,046	20,884	29%
4	1.8	1.66	39,904	37,726	31,664	21%
5	1.8	1.76	37,846	34,578	30,046	21%
6	1.6	1.74	39,866	37,888	32,424	19%
7	1.4	1.61	42,126	39,456	33,872	20%
8	2.3	2.48	33,682	29,334	24,892	26%
9	2.1	2.28	43,478	41,226	37,394	14%
10	2.1	2.09	40,126	36,448	33,282	17%
11	2.1	2.12	31,248	27,170	26,415	15%
12	2.1	1.98	38,848	36,465	29,293	25%
13	2.2	2.06	39,146	37,446	31,709	19%
14	2.2	2.24	27,892	25,028	21,342	23%
15	2.7	2.69	44,610	38,364	31,524	29%
16	1.5	1.44	42,222	40,414	31,244	26%
Mean± SE	2.0 ± 0.3	2.0 ± 0.4	37692 ± 1233	34513 ± 1307	29442 ± 1119	22.2 ± 1.2%

TABLE 6

Recovery of Islets Following Freezing and
Thawing in Presence and Absence of Sertoli Cells

	Islets alone			Islets + Sertoli cells		
No. of islets	Pre-cryo	Post thawing	Recovery (%)	Pre-cryo	Post thawing	Recovery (%)
D3F/D3T	250	152	61%	290	212	73%
	230	131	57%	260	228	88%
	440	278	63%	430	280	88%
	420	366	87%	410	324	79%
	450	290	64%	440	358	81%
		Means	66.4%			81.8%
D7F/D3T	260	136	52%	250	229	92%
	300	208	69%	300	202	67%
	280	177	53%	290	238	82%
	360	205	57%	350	300	86%
	320	218	68%	390	289	74%
	380	217	57%	320	270	84%
		Means	61.0%			80.8%

As shown in Table 5, the yield of islets per gram pancreas was 37692±1233, 34513±1307 and 29,442±1119, after 1, 3 and 7 days of culture, respectively. Following cryopreservation and thawing and reculturing of the cells in the presence of Sertoli cells approximately 20% of the cells were damaged or lost as shown in Table 6. Thus ±24,000 islets/gram of piglet pancreas were available for transplant purposes after cryopreservation and thawing.

The results showed that insulin secretion was blunted when glucose was used as insulin secretagogue prior to cryopreservation. The effect was more evident following cryopreservation and thawing. While the presence of Sertoli cells had marked effects on number of islets that survived cryopreservation and thawing their presence had little effect on the ability of the islets to respond to a low glucose concentration as insulin releasing agent. However, as shown in Example 8 the presence of Sertoli cells augmented the secretion of insulin in the presence of high glucose concentrations and glucose plus Forskolin.

EXAMPLE 5

Response of Diabetic Sprague Dawley Rats to the Transplantation of Islets from Piglet Donors (Discordant Xenografts)

The rats were made diabetic by means of a single i.v. injection of 55 mg/kg of streptozotocin. They were grafted only if the blood sugar was equal to or more than 400 mg/dl. Following transplantation the rats were placed individually in metabolic cages and urine volume, urine glucose content, and body weights were measured at daily intervals. Blood glucose levels were done at weekly intervals. A rat is considered cured of diabetes if the blood glucose level is 160 mg/dl or less and/or the daily urine volume is 15 ml or less.

The results are illustrated in FIGS. **10** and **11**.

FIG. **10** shows the effect of transplantation of piglet islets and Sertoli cells underneath the renal capsule on the mean daily urine output of seven grafted female rat recipients. Each bar represents the mean daily urine output over a ten-day period following transplantation. The study has been conducted over an 80-day period, the bar on the furthest right thus showing the mean urine output per day from day 80 through 89, etc.

The figure shows that the mean daily urine volume for the first 60 days varied between 19.7 mls and 27 mls or within a diabetic range. It can be readily appreciated that urine volumes decreased to near-normal levels only from days 70 through day 89. The corresponding plasma glucose levels during the first and last ten day periods were 474±46 and 155±70, mg/dl, respectively.

These results indicate that following transplantation with piglet islets and Sertoli cells the rats showed evidence of survival of the grafted islets. The reversal to normoglycemia took about 80 days.

It should be noted that one of the cured rats is pregnant and has been normoglycemic throughout her pregnancy.

FIG. **11** shows the effect of the transplantation of piglet islets and Sertoli cells underneath the skin on the mean daily urine volumes of three rats over a 50 day period. The results show that the mean urine volume decreased from a mean of

41.7 ml during the first 10-day period to an average of 12.3 mls during the fifth week. The corresponding glucose levels were 509±45, and 200±12, mg/dl, respectively.

The data depicted above demonstrate that both the renal subcapsular space and the subcutaneous area can be used as a site to create an immunologically privileged site for the transplantation of islet xenografts.

EXAMPLE 6

This study determined the effect of cultured Sertoli cells on the survival of discordant islet xenografts in diabetic rats with minimal early exogenous immunosuppression.

Preparation of Islets

Neonatal piglets of less than seven days of age were killed by anesthesia and islets were isolated according to a method of Kuo C. Y., Burghen G. A., Myvacle A. and Herrod H. G. (1994) "Isolation of islets from neonatal pig pancreatic tissue", *J. Tissue Culture Methods,* 16: 1–7. Briefly, the pancreas was distended by an injection of a collagenase solution, 2 mg/ml, collagenase type XI, in culture medium DMEM. After incubation at 39° C. for 17 min, the digested fragments were washed by centrifugation and the digested tissue was then incubated for one week in medium 199 supplemented with 10% horse serum and 1% antibiotics at 32° C. The islets were then cryopreserved according to the method by Lakey J. R. T., Warnock G. L., Kneteman N. M., Ao Z., Rajotte R. V. (1994) "Effects of precryopreservation culture on human islet recovery and in vitro function", *Transplant Proc.,* 26:820 and stored in liquid nitrogen at −196° C. Three days prior to transplantation the cryopreserved islets were rapidly thawed and cultured at 32° C. for two days. One day prior to transplantation some of the islets were collected and co-cultured with Sertoli cells for 24 hours.

Sertoli cell isolation

Testes of young S-D rats were removed and Sertoli cells were isolated by the method of Cheng C. Y. and Bardin C. W. (1987) "Identification of two testosteroneresponsive proteins in Sertoli cell-enriched culture medium whose secretion is suppressed by cells of the intact seminiferous tubule." *J. Biol. Chem.,* 262:12768–12779. Briefly, the testes were digested first in DMEM containing 1.0% trypsin, and then is DMEM containing 1.0% collagenase, type 1, for periods of 15 min each, at 37° C. The purified Sertoli cells were cultured at 37° C. in DMEM/F12 supplemented with transferrin, 10 ug/ml, FSH 10 ng/ml, insulin 20 ug/ml and 1.0% FCS, for three days. For transplantation, Sertoli cells and islets were pooled and rats were grafted with either a composite consisting of 5×10^6 Sertoli cells and 3,000 islets, or with islets alone (15 islets/g of body weight).

Transplantation of rats

Female S-D rats, weighing between 170 and 200 g were made diabetic by means of a single i.v. injection of 60 mg/kg of streptozotocin. A total of 31 diabetic rats were divided into 3 groups and grafted as follows: Group 1, a control group (n=8), received a total of 15 islets/g body weight injected underneath the renal capsule. No Sertoli cells were grafted. Following transplantation the rats were treated with cyclosporine for 55 days: 25 mg/kg for 3 days, 15 mg/kg for 10 days, 10 mg/kg for 10 days and 5 mg/kg for the following 32 days. Immunosuppression was then stopped. Each rat received, in addition, 1–3 U of Ultralente insulin at daily intervals if the 24-hour urine glucose content exceeded I g. Insulin therapy was stopped on day 55. Group 1, a tissue control group (n=8), was given a renal, subcapsular injection of a composite of about 5×106 Sertoli cells and 3,000 islets. No CsA was given. Insulin was given as depicted above. Group 3, the experimental group (n=15), was transplanted with both sertoli cells and islets and then treated with CsA and insulin according to the schedule outlined above.

Posttransplantation evaluation of rats

Plasma glucose levels were obtained at weekly intervals. Twenty four hour urine volumes and urine glucose contents were recorded daily. A rat was considered cured of the diabetic process if the following criteria applied: A plasma glucose level of equal to or less than 10 mmol/L, a 24-hour urine volume of less than 15 ml, and immediate reversal to hyperglycemia following surgical removal of the grafted kidney. One normoglycemic rat was mated on day 69 to test her ability to become pregnant.

Structural analysis of the grafted tissue

Two normoglycemic rats were nephrectomized on days 117 and 330 and grafted tissue prepared for light and electron microscopy. Selawry H. P., Cameron D. F. (1992) "Sertoli cell-enriched fractions in successful islet cell-transplantation", *Cell Trans.,* 2:123–129. Briefly, tissue wedges were immersion-fixed with 5% glutaraldehyde in 0.1 M collidine buffer for 1 h., washed in buffer, and postfixed for 1 h with 1% osmium tetroxide in 0.1 M buffer. Small tissue blocks were cut from the wedges, and dehydrated through a graded series of ethyl alcohols, transferred to propylene oxide, and embedded in Epon 812/Araldite plastic resin. Thick (0.5 um) and thin (900 ng) sections were stained routinely with toluidine blue and urinal acetate/lead citrate, respectively, for structural analysis by light and electron microscopy.

The results of the effect of Sertoli cells and cyclosporine on survival of xenographic transplantation of pig islet cells into the renal subcapsular space of diabetic female rats are shown in Table 7.

TABLE 7

Group (n)	Sertoli Cells	CsA	Graft Survival (days)
1 (8)	−	=	0, 0, 0, 0, 0, 0, 0, 0
2 (8)	+	−	0, 0, 0, 0, 0, 0, 0, 0
3 (15)	+	+	0, 0, 0, 0, 0, 71, 77, 96, 117* 148#, >154, >165, >327, 330*

*rats nephrectomized to remove the xenograft
#rat died during a cardiac puncture

As shown in Table 7, none of the rats grafted with islets alone and then given CsA and low-dose insulin (Group 1) became significantly less hyperglycemic. Further, none of the rats grafted with a composite of islets and Sertoli cells, but without CsA, showed any improvement of hyperglycemia (Group 2). Of 15 rats grafted with islets and Sertoli cells and then given CsA (Group 3), 10 showed evidence of reversal of the diabetic state. Four of the ten are still normoglycemic for periods of more than 154, 165, 165, and 327 days, respectively. The normoglycemic rats who were nephrectomized on days 117 and 330, became hyperglycemic immediately. Their plasma glucose levels were 4.9 mmol/L, and 8.2 mmol/L, prior to, and 20.7 mmol/L, and 32.2 mmol/L, respectively, following nephrectomy. A female rat who was mated on day 69 became pregnant and delivered a total of 10 pups on day 89, all of whom she nursed successfully while remaining normoglycemic. She died on day 148 as a result of a cardiac puncture. Three of 10 rats regressed into hyperglycemia on days 71, 77, and 96, respectively, after a short period of euglycemia.

These results demonstrate that prolonged survival of a discordant islet xenograft (pig to rat) can be achieved in female diabetic rats. Survival of islet xenografts depended upon two factors which had to be administered concomitantly: Co-transplantation with Sertoli cells and treatment with cyclosporine.

The response of total urine volumes following transplantation with a composite of pig islet and rat Sertoli cells measured at 10-day intervals over an 80 day period for 7 of

the improved rats showed an average daily urine volume of 27.0±13.0 ml/rat during the first 10-day period, which slowly declined to a mean of 12.0±4.0 ml/rat, 70 days following transplantation.

Tissue morphology studies shown in FIG. 12 show that the tissue and cellular structure of kidney parenchyma appeared normal in the rat nephrectomized 117 days following transplantation. Normal appearing islets with structurally distinct B-cells were visible in well vascularized areas subjacent to the kidney capsule. Additionally, normal appearing Sertoli cells were observed adjacent to the transplanted islets along with numerous lymphocytes. No plasma cells were identified at the transplantation site. Viable endocrine cells were similarly observed in the subcapsular renal space of the rat nephrectomized 330 days following transplantation.

These studies show that significant prolongation of survival of a discordant islet xenograft can be achieved without sustained immunosuppression. These studies demonstrate that the mechanism by which Sertoli cells promote islet xenograft survival is three-fold: (1) Sertoli cells stimulate the recovery of islets damaged during transplantation (i.e. improve the yield and function of cultured islets), (2) Sertoli cells protect grafted islets from immunologic rejection by producing factors which strongly suppress proliferation of T-cells, and (3) Sertoli cells protect grafted islets from the toxic effects of cyclosporine.

EXAMPLE 7

This study shows a method of isolating and cryopreserving porcine pancreatic islets for future xenographic transplants in mammals.

Male piglets, <7 days old and weighing 2± kg were used as donors. The pancreases, weighing 1.4±0.3 g, were harvested and injected with DMEM solution containing 2 mg/ml collagenase XI. The distended pancreas was incubated in a shaking water bath at 39° C. for 17 min. The digested tissue was filtered through a 500 μm stainless steal filter and filtrates were washed×3 with cold DMEM. Without further purification the cells were cultured in M199 and 10% horse serum at 32° C. for 7 days. The islet cells were then cryopreserved using standard procedures. At specified intervals islets were thawed and cultured in M199, both in presence, and isolated from testes of male piglets according to a standard method.

To test functional capacity, islets cultured for 3 and 7 days were assessed for insulin release in static incubation. In separate experiments, effect of insulin secretagogues was tested on islets cultured with and without Sertoli cells. The results of this study are shown in Tables 8 and 9.

TABLE 8

Effect of Insulin Secretagogues, Glucose and Glucose Plus Forskolin, on Insulin Release From Incubated and Frozen/Thawed (F/T) Islets in the Presence and Absence of Pig Sertoli Cells

	Insulin Release (uU/ml/10 islets)		
	3.3 mmol/L glucose	16.7 mmol/L glucose	16.7 mmol/L glucose +100 umol Forskolin
Day 3 Incubated with Sertoli cells	42.3 ± 1.2	112.8 ± 17.7*#	267.7 ± 43.0**#
Day 3 Incubated alone	31.3 ± 2.1	57.3 ± 3.8*	123.4 ± 15.3**

TABLE 8-continued

Effect of Insulin Secretagogues, Glucose and Glucose Plus Forskolin, on Insulin Release From Incubated and Frozen/Thawed (F/T) Islets in the Presence and Absence of Pig Sertoli Cells

	Insulin Release (uU/ml/10 islets)		
	3.3 mmol/L glucose	16.7 mmol/L glucose	16.7 mmol/L glucose +100 umol Forskolin
Day 7 Incubated with Sertoli cells	22.9 ± 1.9	64.5 ± 6.4*#	153.9 ± 14.6**
Day 7 Incubated alone	21.3 ± 1.2	37.3 ± 6.0*	120.3 ± 11.4**
Day 3 F/T with Sertoli cells	20.6 ± 4.3	44.9 ± 9.9*	77.1 ± 13.7**
Day 3 F/T alone	11.7 ± 2.3	27.9 ± 6.6*	54.5 ± 10.7**

Anova Test: *vs 3.3 mmol/L p 0.5, **vs both 3.3 & 16.7 mmol/L P < 0.05
#with Sertoli cells vs islets alone P < 0.05

TABLE 9

Effect of Sertoli cells on insulin content of incubated and frozen-thawed piglet islets.

	Insulin content (uU/10 islet(s)	
	Islets alone	Islets & Sertoli cells
Incubated D1	257.0 ± 19.6	391.1 ± 51.4*
Incubated D3	201.1 ± 19.1#	400.1 ± 41.0*#
Incubated D7	179.1 ± 26.2#	271.9 ± 39.9*#
Frozen D3/Thaw D3	52.4 ± 10.3	132.5 ± 35.1
Frozen D7/Thaw D3	10.4 ± 0.9	35.1 + 8.2

Anova *islets +Sertoli cell vs. islet alone P < 0.05
#Incubated islets D3, D7 vs. Frozen D3, D7 P < 0.05

These results show that: (1) large numbers of neonatal porcine islets can be isolated by a simple method; (2) cryopreservation and thawing results in about 40% loss in number of islets in the absence of Sertoli cells and about a 20% loss in the presence of Sertoli cells; (3) cultured islets have the ability to respond to both glucose and glucose+ Forskolin as insulin secretagogues; (4) the functional capacity of the cocultured islet was enhanced two-fold in the presence of Sertoli cells; (5) following cryopreservation and thawing, islets recover more rapidly in presence of Sertoli cells and the response to both glucose and glucose+ Forskolin was enhanced two fold in the presence of Sertoli cells.

EXAMPLE 8

This example describes a method of treating genetic diabetes which is demonstrated using the animal model NOD (non-Obese Diabetic).

Genetic diabetic mice (NOD) are recipients of pancreatic islet xenografts. Diabetic mice are selected from the colony of NOD mice maintained at the University of Tennessee Medical Center. The current incidence of diabetes in this colony is 80% for females and 63% of males by 25 weeks of age. Animals are considered diabetic if they have two consecutive weekly urine glucose readings of ½% (3+) and a confirmatory plasma glucose greater than 400 mg/dl. Three to ten days before transplantation of the graft, the animals receive appropriate insulin to stabilize their health. Diabetic animals are randomly divided into two groups. All animals in these two groups receive 0.3 mg of the antiCD4 antibody,

GK1.5 on days −1, 0 and 1 to initiate immunosuppression. Maintenance immunosuppression with GK1.5 cyclosporine A, FK506, cyclophosphamide, rapamycin, nicotinamide or 15-deoxyspergualin may be required.

Porcine pancreatic islets for transplantation are prepared as described in Example 6 except that they are not cryopreserved. Nicotinamide (10 mM) or IGF-1 may be added to the incubation medium prior to transplantation. Porcine sertoli cells for transplant are prepared as described in Example 6. Cyclosporine may be included in the culture medium during the first four incubation days prior to transplantation.

One group of the diabetic NOD mice receive 3,000 porcine islets in 25 μl of Hank's buffered salt solution (HBSS) under the right renal capsule followed by an injection of 2×10^7 pig Sertoli cells in 25 μl in HBSS under the same renal capsule. A second group of mice receive a transplant consisting of only the porcine islet cells. Animals with xenografts continue to receive daily insulin injections; the amount determined by the concentration of glucose in the animals urine and plasma. Mice with plasma glucose levels less than 250 mg/dl are considered cured and no additional insulin administered.

A majority of mice receiving porcine sertoli cells and porcine pancreatic islets attain normal urine and plasma glucose levels (xenograft acceptors). In contrast, the majority of mice receiving porcine islets alone exhibit graft failure and do not attain normal glucose levels in the urine or plasma.

EXAMPLE 9

This example shows a method of monitoring the immune response elicited against cellular transplants.

Total spleen leukocytes are isolated from mice by methods known in the art from mice that have rejected their porcine islet xenografts. In preliminary experiments these leukocytes are administered to NOD/scid mice (NOD mice that in addition have genetic immunodeficiency) with successful pig islet xenografts. The leukocytes cause rejection of the xenograft.

Plastic adherence (to deplete macrophages), nylon wool adherence (to deplete non-T lymphocytes), or specific antibodies (anti-CD4, anti-CD8, anti-F4/80, anti-B220) are used to deplete the total splenocyte preparations of certain classes of leukocytes. The class-depleted leukocyte preparations are injected into the NOD/scid mice with successful pig islet xenografts to determine which leukocyte class is necessary and sufficient to cause xenograft rejection.

Spleen leukocytes are isolated by conventional methods known in the art from mice (xenograft acceptor of Example 8) that have accepted their pig islet xenografts. These leukocytes are administered to NOC/scid mice with successful pig islet xenografts. The leukocytes do not cause rejection of the xenograft. Combining the appropriate leukocyte-depleted preparation that causes rejection with leukocytes from a xenograft acceptor of Example 8 (50/50 mixture) and administering the mixed cell population to NOD/scid xenograft recipients (adoptive transfer) allows determination of whether so-called suppressor lymphocytes are preventing xenograft rejection in xenograft acceptors.

EXAMPLE 10

This example provides a method of encapsulation of Sertoli cells with islets for transplantation.

Preparation of Islets and Sertoli Cell Isolation

Islets are isolated from female Fischer rats and are incubated in CMRL medium for approximately four days prior to usage. Sertoli cells are isolated from weanling male Fischer rats and are incubated for approximately four days to confluency in Petri dishes at 37° C.

After four days of incubation, the islets are counted and groups of 50 islets are transferred to 24 well Petri dishes. Sertoli cells are removed from Petri dishes with Sigma non-enzymatic media. The Sertoli cells are washed and counted.

Three experimental groups are then established as follows: Group 1: 12-well Petri dishes, each well containing 50 islets; Group 2: 12-well Petri dishes, each well containing a total of 1×10^4 Sertoli cells; and Group 3: 12-well Petri dishes, each well containing 50 islets, plus 1×10^4 Sertoli cells. The Petri dishes are incubated at 37° C. for 24 hours to permit Sertoli cells to attach to islets.

Microencapsulation of Islets and Sertoli Cells

Islets, Sertoli cells and islets plus Sertoli cells are encapsulated by suspension in a solution of sodium alginate which is sprayed into a dish of calcium chloride using a droplet forming device according to the method of Lim et al. (1980 Science 210:908–910, the contents of which is incorporated by reference. The droplets are coated with a layer of poly-L-lysine (PLL) with an average size of 20 kDa at a concentration of 0.05% (w/v) and a reaction time of 6 minutes according to the method of Goosen et al. (1985) Biotechnol. Bioeng. 27:146–150, the contents of which is incorporated by reference. An additional outer layer of sodium alginate is added around the capsule according to the method of O'Shea et al. (1984) Biochim. Biophys. Acta 804:133–136, incorporated herein by reference. Alternatively, isolated cells may be encapsulated according to the methodology of Weber et al. U.S. Pat. No. 5,227,298, incorporated herein by reference. Following encapsulation, the cells are divided into treatment groups. Group 1: Free islets, not encapsulated, Group 2: Islets alone, encapsulated, Group 3: Sertoli cells alone, encapsulated, and Group 4: Islets plus Sertoli cells encapsulated. Encapsulated cells are placed in media conventionally selected by the skilled artisan at 37° C.

In Vitro Encapsulation

At specified intervals following encapsulation, i.e., 1, 7, 14, 21 and 30 days respectively, following incubation, functionality of the groups containing islets are examined. Approximately 10 capsules from each of the islet containing groups are stimulated, in tandem, by a buffered medium containing glucose 9 mmol/L, glucose at 16.7 mmol/L, and Forskolin at 10 mM for 30 minutes, in a water bath at 37° C. The perfusate is collected and insulin is assayed using a commercially available kit (e.g., Linco insulin kit). Insulin content of free encapsulated islets and islets encapsulated with Sertoli cells is further examined via an acid-ethanol extract of said capsules and assayed for insulin content using a commercially available kit (e.g. Linco insulin kit).

In Vivo Encapsulation

Female Wistar-Furth rats are made diabetic by means of a single i.v. injection of streptozotocin. A total of 32 diabetic rats are divided into four groups and treated as follows: Group 1, a control group (n=8), will receive an intraperitoneal injection of at least 10 capsules containing Sertoli cells alone; Group 2 (n=8) will receive an intraperitoneal injection of 10 islets/g of free, non-encapsulated islets; Group 3 (n=8) will receive an i.p. injection of 10 islets/g of encapsulated islets alone; Group 4, (n=8) will receive 10 islets/g of co-encapsulated islets plus Sertoli cells. No group will receive any immunosuppression following transplantation. All rats are closely monitored via daily plasma glucose levels for the first week post-transplantation, and then at weekly intervals thereafter.

Rats are considered cured of diabetes if they exhibit a blood glucose level less than or equal to 170 mg/dl with concomitant steady increases in body weight.

EXAMPLE 11

This example describes a method of immortalizing Sertoli cells using a temperature-sensitive mutant of the SV40 virus.

Sertoli cells are isolated from sexually mature (120 days) rats according to the method of Wright et al. (1989) Ann. NY Acad. Sci. 564:173–185. The testes are removed and the tubules are resuspended in DMEM F-12 (DMEM/F12: Life Technologies, Inc., Grand Island, N.Y.) containing 1 mg/ml collagenase (Worthington, Freehold, N.J.), 2mg/ml hyaluronidase (Sigma, St. Louis, Mo.), 0.3 mg/ml DNase (Sigma), and 65 ug/ml soybean trypsin inhibitor (Sigma), and are incubated for 25 min at 32° C. with gentle shaking. The incubation is repeated and the tubules are washed in F12/DMEM and digested in an enzyme solution including 1 mg/ml collagenase/dipase (Boehringer-Mannheim, Indianapolis, Ind.) in lieu of collagenase. The tubules are recovered and further broken up by gentle pipetting with a Pasteur pipette. The digestion mixture is filtered through a nylon mesh to remove clumps of undispersed Sertoli cells. The Sertoli cells are then sedimented at unit gravity yielding a 95% pure population of adult Sertoli cells.

Two 25-cm^2flasks are seeded with Sertoli cells at a density of 5×10^6 cells per plate. These flasks are incubated with SV40 virus mutant tsA255 for 3 h. The virus-containing medium is removed, and the cells are incubated at 33° C. in F12/DMEM supplemented with 4% fetal bovine serum (FBS). Foci of transformed cells are visible at 6 weeks after infection. Each individual focus is isolated with sterile metal rings, the cells are isolated from the plate with trypsin EDTA, and the cell suspensions are replated in 25-cm^2 flasks. Aliquots of cells from each focus are cultured at 33° C. or 40° C. for two days. At the end of this culture period, the cells are collected and total RNA is isolated for Northern blot analysis according to the methodology of Roberts, et al. (1992) Biol. Reprod. 47:92–96, incorporated herein by reference. Sertoli cells are selected for cloning on the basis of the inducible expression of mRNAs encoding Sertoli cell-secreted proteins according to the methods of Roberts, et al. (1995) Biol. Reprod. 53:1446–1453, incorporated herein by reference. Clonal cells are cultured in DMEM/F12 Plus 4% FBS, supplemented with 1% antibiotic-antimycotic. The cells are then seeded and allowed to attach at 33° C. for at least 24 hours. Clonal cells are collected by washing the plates twice with Hanks balanced salt solution followed by a brief incubation with trypsin/EDTA.

What is claimed is:

1. A method of treating a disease that results from a deficiency of a biological factor in a mammal wherein said method comprises administering Sertoli cells obtained from a cell line and a therapeutically effective amount of cells that produce said biological factor to a mammal in need of such treatment, wherein said Sertoli cells are administered in an amount effective to create an immunologically privileged site.

2. The method of claim 1 wherein said mammal is a human.

3. The method of claim 1 wherein said biological factor is a hormone.

4. The method of claim 1 wherein said biological factor is insulin and said disease is diabetes mellitus.

5. The method of claim 4 wherein said cells that produce said biological factor are pancreatic islet of Langerhans cells.

6. The method of claim 1 wherein said cells that produce said biological factor are cells transformed by a nucleic acid encoding said biological factor.

7. The method of claim 1 wherein said administering is by transplantation.

8. The method of claim 1 wherein said Sertoli cells are administered in a dosage ranging from 10^5 to 10^{10} cells.

9. The method of claim 1 wherein said cells that produce said biological factor are administered in a dosage of from 10^5 to 10^{10} cells.

10. The method of claim 7 wherein said transplantation is by xenograft.

11. The method of claim 7 wherein said transplantation is by allograft.

12. The method of claim 1 which further comprises administering an immunosuppressive agent.

13. The method of claim 12 wherein said immunosuppressive agent is administered for a time sufficient to permit said transplanted cells to be functional.

14. The method of claim 12 wherein said immunosuppressive agent is cyclosporine.

15. The method of claim 14 wherein said cyclosporine is administered at a dosage of from 5 to 40 mg/kg body wt.

16. The method of claim 1 which further comprises administering a therapeutically effective amount of exogenous biological factor following the transplantation of said cells that produce said biological factor.

17. The method of claim 1 wherein said cells that produce said biological factor are co-cultured with Sertoli cells in tissue culture.

18. The method of claim 17 wherein said cells that produce said biological factor are cryopreserved prior to co-culturing with Sertoli cells in tissue culture.

19. The method of claim 1 wherein said Sertoli cells are obtained by the steps comprising:

(a) isolating mammalian Sertoli cells from mammalian tissue;

(b) incubating said isolated mammalian Sertoli cells with virus producing cells under conditions sufficient to transform said Sertoli cells; and

(c) isolating said transformed cells from the virus producing cells.

20. The method of claim 1 wherein said Sertoli cells are obtained by the steps comprising:

(a) isolating mammalian Sertoli cells from mammalian tissue;

(b) incubating said isolated mammalian Sertoli cells with a mutagen under conditions sufficient to transform said Sertoli cells; and

(c) collecting said transformed Sertoli cells.

21. The method of claim 19, wherein said virus producing cells are SV40 or polyoma virus.

22. A method of treating diabetes mellitus in a mammal wherein said method comprises administering to a diabetic mammal Sertoli cells obtained from a cell line in an amount effective to create an immunologically privileged site and a therapeutically effective amount of pancreatic islet of Langerhans cells.

23. The method of claim 22 wherein said diabetes mellitus is type I or type II.

24. The method of claim 22 wherein said mammal is a human.

25. The method of claim 22 wherein said Sertoli cells are human, bovine or porcine.

26. The method of claim 22 wherein said pancreatic islet of Langerhans cells are human, bovine or porcine.

27. The method of claim 22 wherein said administering is by transplantation.

28. The method of claim 27 wherein said transplantation is by injection into the renal subcapsular space.

29. The method of claim 27 wherein said transplantation is by injection into the subcutaneous facie.

30. The method of claim 22 wherein said Sertoli cells are administered at a dosage ranging from 10^5 to 10^{10} cells.

31. The method of claim 22 wherein said islet of Langerhans cells are administered at a dosage ranging from 5–1000 islet cells/g body wt.

32. The method of claim 22 which further comprises the administration of an immunosuppressive agent.

33. The method of claim 32 wherein said immunosuppressive agent is administered for a time sufficient to permit the transplanted islets to be functional.

34. The method of claim 32 wherein said immunosuppressive agent is cyclosporine.

35. The method of claim 32 wherein said cyclosporine is administered at a dosage of 5 to 40 mg/kg body wt.

36. The method of claim 22 which further comprises administering a therapeutically effective amount of insulin following transplantation of said pancreatic islet of Langerhans cells.

37. The method of claim 22 wherein said Sertoli cells are obtained by the steps comprising:

(a) isolating mammalian Sertoli cells from mammalian tissue;

(b) incubating said isolated mammalian Sertoli cells with virus producing cells under conditions sufficient to transform said Sertoli cells; and

(c) isolating said transformed Sertoli cells from the virus producing cell.

38. The method of claim 22 wherein said Sertoli cells are obtained by the steps comprising:

(a) isolating mammalian Sertoli cells from mammalian tissue;

(b) incubating said isolated mammalian Sertoli cells with a mutagen under conditions sufficient to transform said Sertoli cells; and

(c) collecting said transformed Sertoli cells.

39. The method of claim 37 wherein said virus producing cells are SV40 or polyoma virus.

40. A method of treating an autoimmune disease in a mammal wherein said method comprises transplanting in to said mammal a therapeutically effective amount of isolated Sertoli cells obtained from a cell line to a transplant site in said mammal having said autoimmune disease, wherein said site is other than testes.

41. The method of claim 40 wherein said Sertoli cells are administered in a dosage ranging from 10^5 to 10^{10} cells.

42. The method of claim 40 wherein said Sertoli cells are obtained by the steps comprising:

(a) isolating mammalian Sertoli cells from mammalian tissue;

(b) incubating said isolated mammalian Sertoli cells with virus producing cells under conditions sufficient to transform said Sertoli cells; and

(c) collecting said transformed Sertoli cells from the virus producing cell.

43. The method of claim 40 wherein said Sertoli cells are obtained by the steps comprising:

(a) isolating mammalian Sertoli cells from mammalian tissue;

(b) incubating said isolated mammalian Sertoli cells with a mutagen under conditions sufficient to transform said Sertoli cells; and

(c) isolating said transformed Sertoli cells.

44. The method of claim 42 wherein said virus producing cells are SV40 or polyoma virus.

45. A method of enhancing the recovery and proliferation of ex vivo cells comprising co-culturing said cells with Sertoli cells for a time and under conditions sufficient to achieve said enhanced recovery and proliferation.

46. A method of enhancing the recovery and proliferation of ex vivo cells comprising culturing said cells in a tissue culture medium containing Sertoli cells for a time and under conditions sufficient to achieve said enhanced recovery and proliferation.

47. A method of treating a disease that results from a deficiency of a biological factor in a mammal wherein said method comprises administering Sertoli cells and a therapeutically effective amount of cells that produce said biological factor to a mammal in need of such treatment, wherein said Sertoli cells are administered in an amount effective to create an immunologically privileged site; and wherein said Sertoli cells and said cells that produce a biological factor are co-localized.

48. The method of claim 47 wherein said biological factor producing cells are co-localized by co-encapsulation.

49. The method of claim 47 or 48 wherein said disease is diabetes mellitus and said biological factor producing cells are islet of Langerhans cells.

50. The method of claim 19, 20, 37, 38, 42 or 43 wherein said transformed Sertoli cells are screened for expression of an appropriate isolate for cloning.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,958,404
DATED : September 28, 1999
INVENTOR(S) : Helena P. Selawry et al.

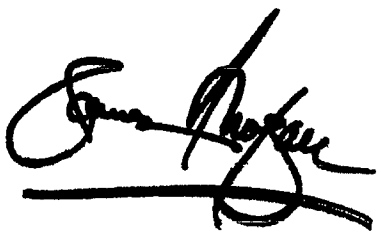
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 20,
Table 3, ">422" should read -- >422t --

Column 21,
Line 21, "of" should read -- Of --

Signed and Sealed this

Fifth Day of August, 2003



JAMES E. ROGAN
Director of the United States Patent and Trademark Office